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13. ABSTRACT (Maximum 200 Words)

Excessive consumption of dietary fat may enhance the rate of breast cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Among different adhesion molecules, evidence indicates that intracellular adhesion molecule-1 (ICAM-1) may play a critical role in breast cancer metastatic formation. Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) expression through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis.

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1. INTRODUCTION

Dietary fat is considered to be one of the main risk factors of carcinogenesis. For example, a positive correlation was reported between dietary fat intake and increased risks for the development of breast, colon and prostate cancers. However, it should be noted that the role of dietary fat in the development of human breast cancer has recently been questioned. Although data obtained from animal studies, international correlation analyses, and meta-analysis of dietary fat intervention studies strongly indicate the association between fat consumption and the development of breast cancer, data from prospective cohort studies on dietary fat and breast cancer suggested that dietary fat might not be a risk factor for human breast cancer. Among different dietary fatty acids, it appears that linoleic acid (C18:2, ω-6) can promote carcinogenesis. In addition to its role in carcinogenesis, dietary linoleic acid can also enhance the metastatic formation of mammary tumors. For example, a linoleic acid-enriched diet increased the rate of metastasis of mammary cancer to the lung in rats.

Excessive consumption of dietary fat may also enhance the rate of cancer metastasis. In addition, it is generally accepted that the upregulation of endothelial cell adhesion molecules is involved in the formation of blood-borne metastasis. Such a process may initiate migration of tumor cells through the endothelium into underlying tissues and thus tumor cells cannot be destroyed by the immune system.

The formation of blood-borne metastasis is a complex process which requires several steps. However, a growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical event in metastasis formation (16,17). This process requires the binding of tumor cells to specific adhesion molecules on the surface of endothelial cells, followed by migration of tumor cells through the endothelium into underlying tissues (16). Evidence indicates that among several adhesion molecules which can be involved in this process, intracellular adhesion molecule-1 (ICAM-1) may play one of the most important roles.

In our research we are the first to propose that lipid-enhanced breast cancer metastasis may be connected to the overexpression of ICAM-1. The fact that a variety of fatty acids have different effects on ICAM-1 induction may explain different effects of dietary lipids on breast cancer metastasis. In the current grant application, we propose to study mechanisms of lipid-induced ICAM expression and breast tumor cell metastatic formation on molecular, cellular and whole animal levels.

Recent evidence indicates that also other inflammatory mediators, namely vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) can be involved in fatty acid-induced cancer metastasis. Therefore, our research has been extended to study effects of dietary lipids, primarily linoleic acid on the expression of VCAM-1 and MCP-1.

2. BODY

a. Research accomplishments associated with Task 1.

Task 1. **To identify the specific phosphorylation mechanism involved in lipid-mediated induction of ICAM-1 expression.**

The results obtained as a result of this Task indicate that exposure to linoleic acid increases protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activities. In addition, inhibition of both PKC and MAP-kinase prevented linoleic acid-mediated activation of

NF-κB. Endothelial cell exposure to linoleic acid also decreased cAMP levels, which indicates that c-AMP-dependent protein kinase (PKA) is an unlikely participant in fatty acid-mediated activation of NF-κB. Thus, in this research we identified two specific signal transduction mechanisms responsible for fatty acid-mediated activation of NF-κB. Which of these two pathways plays more important role in fatty acid-mediated activation of NF-κB and ICAM-1 gene expression requires further studies. Such studies may involve transfections of endothelial cells with specific NF-κB as well as IκB reporter contracts. Because endothelial cells are well known to be difficult to transfect, we developed a special technique which allows us to achieve a high-efficiency transfection of human endothelial cells. This technique was recently published by our group (Kaiser and Toborek J. Vasc. Res. 38:133-143, 2001) and it constitutes another major accomplishment resulting from this grant proposal. We were the first to report that transfection of endothelial cells can achieve as high as 32% efficiency (Figure 1). This technique also was employed in our research on NF-κB-mediated induction of ICAM-1 gene, as well as other inflammatory genes in human endothelial cells (Toborek et al., Am. J. Clin. Nutr. 75, 119-125, 2002; Lee et al., J. Nutr. Biochem. 12, 648-654, 2001; Park at al., Nutr. Cancer, 41, 126-134, 2001).

Detailed descriptions of the obtained results are included in the appended publications.

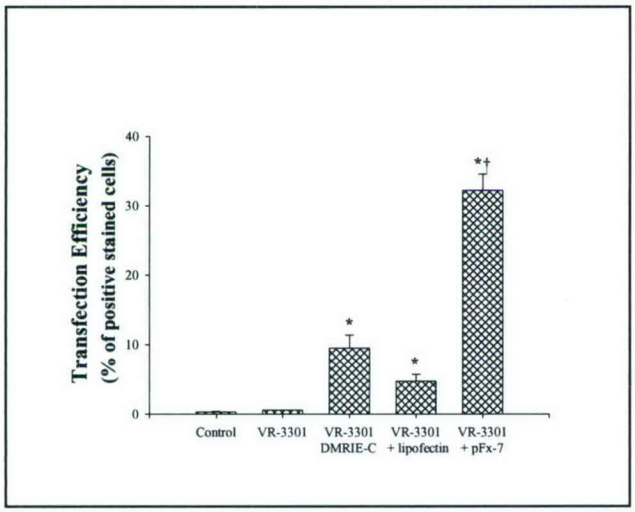

Figure 1. Efficiency of liposome-mediated transfection of human endothelial cells. Cells were transfected for 1.5 h with the VR-3301 vector (5 μg/mL) complexed with 40 μg/mL of DMRIE-C or lipofectin or with 36 μg/mL of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups.

b. Research accomplishments associated with Task 2.

Task 2. **To test the hypothesis that induction of ICAM-1 expression mediated by polyunsaturated but not saturated fatty acids, is the critical factor in promoting adhesion of breast tumor cells to endothelial cells and their transendothelial migration.**

Extensive studies were performed in relationship to this Task. We indicated that dietary fatty acids can exert specific effects on ICAM-1 gene expression. Exposure to both linoleic acid and linolenic acid induced a dose dependent increase in ICAM-1 mRNA levels. In addition, these two fatty acids at the concentration of 90 μmol/L stimulated induction of the ICAM-1 gene to a similar extent, i.e., by approximately 30% as measured by the density of the appropriate fluorescent bands. In contrast, exposure of endothelial cells to oleic acid decreased ICAM-1 mRNA levels to approximately 50% of control values. The results of these experiments are shown in Figure 2. The full report on dietary fatty acid-mediated expression of inflammatory

of Clinical Nutrition (Toborek et al., Am. J. Clin. Nutr. 75, 119-125, 2002) and is also appended to this Progress Report.

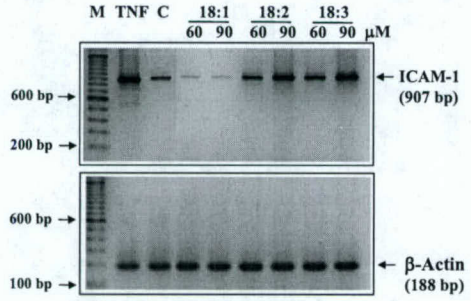

Figure 2. Effects of dietary fatty acids on intercellular adhesion molecule-1 (ICAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determined to indicate that the same amount of RNA was used per sample.

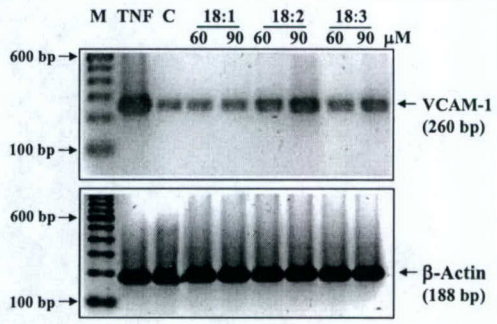

Figure 3. Effects of dietary fatty acids on vascular cell adhesion molecule-1 (VCAM-1) mRNA levels in human endothelial cells as measured by RT-PCR. Endothelial cells were exposed to specific fatty acids for 3 hours. β-Actin was determined to indicate that the same amount of RNA was used per sample.

It appears that not only ICAM-1 but also another adhesion molecule, namely vascular cell adhesion molecule-1 (VCAM-1), may play an important role in dietary fatty acid-mediated cancer metastasis. The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are indicated in Figure 3. The most significant induction of the VCAM-1 gene (by 38% as measured by the density of the fluorescent bands) was observed in cells treated with 90 μmol/L of linoleic acid. In addition, exposure to 90 μmol/L of linolenic acid resulted in a slight increase in VCAM-1 mRNA levels. Treatment with oleic acid had no effect on VCAM-1 gene induction as compared to control cultures.

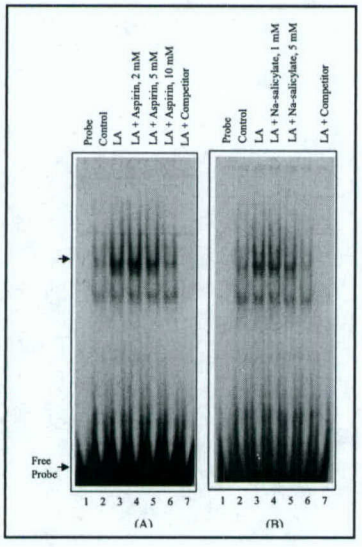

Figure 4. Pretreatment with aspirin, sodium salicylate or PDTC blocks linoleic acid (LA)-induced NF-κB DNA-binding activity in human microvascular endothelial cells (HMEC-1) as measured by EMSA. HMEC-1 were pretreated for 1 h with indicated concentrations of (A) aspirin or (B) sodium salicylate before a 2 h treatment with 50 μM of linoleic acid (lanes 4-6). Lane 1, probe alone; lane 2, treatment with 50 μM linoleic acid alone; lane 7, competition study performed by the addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 μM linoleic acid.

Our research on fatty acid-induced inflammatory responses in human endothelial cells gained significant national and international attention and recognition. Our paper on this subject published in the American Journal of Clinical Nutrition was accompanied by the Editorial Comments and has been featured on several news releases, such as Reuters Health and the Medical Post of Canada (please see the appended material).

Because of the profound effects of linoleic acid on VCAM-1 gene expression, detailed studies were performed on the mechanisms of this process. We indicated that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in human endothelial cells. In addition, we indicated that common anti-inflammatory drugs, such as aspirin or sodium salicylate can inhibit linoleic acid-mediated activation of NF-κB (Figure 4) as well as linoleic acid-induced VCAM-1 expression (Figure 5).

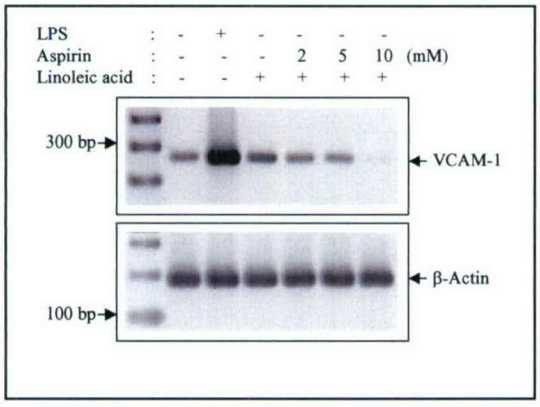

Figure 5. Pretreatment with aspirin impedes the induction of VCAM-1 mRNA expression in linoleic acid-treated human microvascular endothelial cells (HMEC-1). Cells were pretreated for 1 h with indicated concentrations of aspirin for 30 min with PDTC, before a 4 h treatment with 50 μM of linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 μg/mL) was used as positive control.

b. Research accomplishments associated with Task 3.

Task 3. **To test the hypothesis that diets enriched with polyunsaturated dietary fats but not saturated fats increase metastasis formation and breast tumor development in an animal model by induction of ICAM-1 expression.**

Our new data indicate that feeding animals with high-fat diets, especially diets enriched in linoleic acid can lead to overexpression of inflammatory genes, such as ICAM-1 and VCAM-1 (Figures 6 and 7).

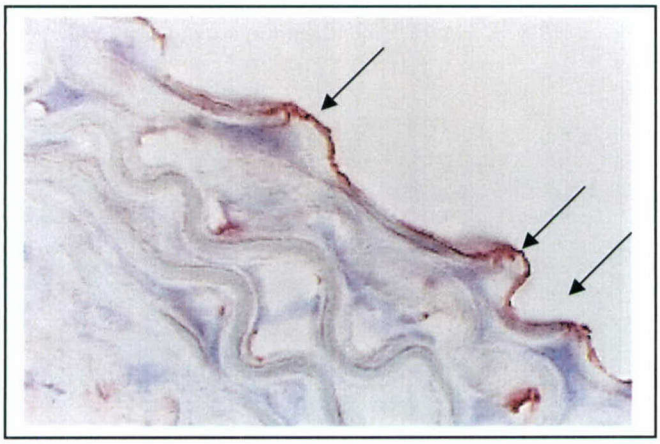

Figure 6. Overexpression of ICAM-1 in the vascular endothelium (arrows) of mice fed high-fat diets. In contrast, the control group does not show positive ICAM-1 immunoreactivity (data not shown).

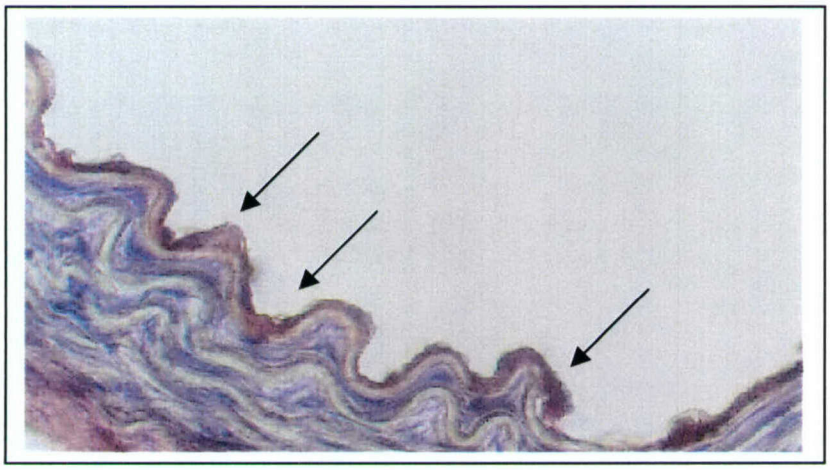

Figure 7. Overexpression of VCAM-1 in the vascular endothelium (arrows) of mice fed high-fat diets. In contrast, the control group does not show positive ICAM-1 immunoreactivity (data not shown).

Overexpression of inflammatory responses was associated with accumulation of macrophages in the vessel wall. The representative image is shown in Figure 8.

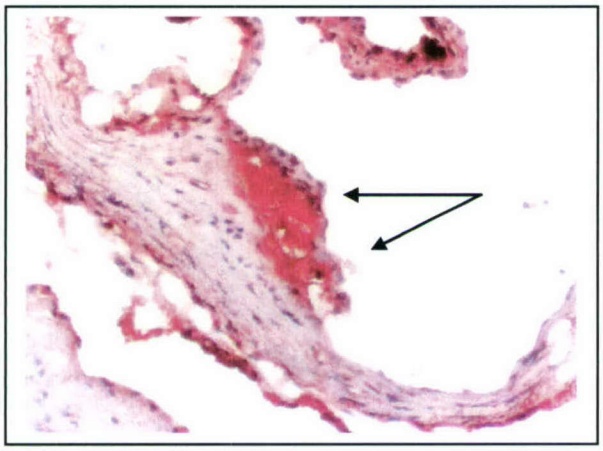

Figure 8. Accumulation of macrophages (arrows) in the aortic wall of mice fed high-fat diet. Macrophage accumulation was correlated with overexpression of adhesion molecules such as ICAM-1 and VCAM-1.

Based on experiments illustrated in Figures 5-8, we completed studies in which nude mice were fed high-fat diets and injected with the MDA-MB-231 metastatic breast cancer cells. The animals injected with the MDA-MB-231 cells developed increased mortality and the development of metastases as compared to control mice. The specific images from these experiments are currently being analyzed.

3. KEY RESEARCH ACCOMPLISHMENTS

- To identify two specific phosphorylation pathways which are induced by dietary fatty acids and participate in fatty acid-mediated activation of NF-κB.
- To establish a new transfection technique which allows the transfection of human endothelial cells with a high efficiency.
- To indicate that antioxidants and common anti-inflammatory drugs, such as aspirin, can inhibit dietary fatty acid-mediated activation of NF-κB and adhesion molecule expression in endothelial cells.

- To determine that the NF-κB binding site plays the critical role in linoleic acid-induced expression of adhesion molecules in human endothelial cells.
- To identify that a high-fat diet can contribute to increased formation of cancer metastases. Formation of cancer metastases was correlated with increased cell adhesiveness into vascular endothelium.

4. REPORTABLE OUTCOMES

a. REFEREED ARTICLES

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5. CONCLUSIONS

Our studies have demonstrated that dietary fatty acids can exert highly specific effects on NF-κB activation and expression of adhesion molecules in human endothelial cells. In addition, we indicated that linoleic acid induces ICAM-1 and VCAM-1 expression through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because ICAM-1 and VCAM-1 are considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. These studies also provide a mechanistic insight of the role of specific dietary lipids in metastasis. Therefore, data arising from this grant proposal may allow dietary and molecular intervention to protect against breast cancer metastasis.

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VEGF regulates PCB 104-mediated stimulation of permeability and transmigration of breast cancer cells in human microvascular endothelial cells

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Abstract

Polychlorinated biphenyl (PCB) congeners, a group of worldwide, persistent environmental contaminants, are known to cause carcinogenesis and tumor promotion, and may also affect the development of cancer metastasis. Because vascular endothelial cells create a selective barrier to the passage of cancer cells, we hypothesize that specific PCB congeners can disrupt endothelial integrity and increase the transendothelial migration of tumor cells. To examine this hypothesis, we elucidated the effects of 2,2′,4,6,6′-pentachlorobiphenyl (PCB 104), a representative of highly *ortho*-substituted non-coplanar PCB congeners, on the endothelial permeability and transendothelial migration of MDA-MB-231 breast cancer cells. Exposure of human microvascular endothelial cell 1 (HMEC-1) to PCB 104 induced endothelial hyperpermeability and markedly increased transendothelial migration of MDA-MB-231 cells. These effects were associated with overexpression of vascular endothelial growth factor (VEGF). PCB 104-mediated elevation of VEGF expression was induced by phosphatidylinositol 3-kinase (PI3K) but not affected by co-treatments with antioxidants or the NF-κB inhibitor SN50. In addition, the PI3K-dependent pathway was involved in PCB 104-induced activation of AP-1, a transcription factor implicated in the regulation of VEGF gene expression. The VEGF receptor (KDR/Flk-1) antagonist SU1498 and the PI3K inhibitor LY294002 inhibited PCB 104-induced hyperpermeability. These results indicate that PCB 104 may contribute to tumor metastasis by inducing VEGF overexpression that stimulates endothelial hyperpermeability and transendothelial migration of cancer cells.

Keywords: PCB 104; VEGF; Metastasis; Endothelial cells; Permeability; Breast cancer

Introduction

During the formation of blood-borne metastasis, tumor cells disseminate from the primary tumor to secondary sites in various organs. Because vascular endothelial cells form a continuous monolayer which functions as a selective barrier to the passage of cancer cells from blood stream to the underlying tissues, endothelial dysfunction has a significant influence on the fate of circulating cancer cells in the blood vessel [1–3]. Specifically, an increase in endothelial permeability can accelerate metastatic process through the facilitated transmigration of cancer cells across the microvascular endothelial monolayer [4–6].

Endothelial hyperpermeability can be induced either by intracellular or extracellular stimuli such as reactive oxygen species (ROS), cytokines, and growth factors [7–9]. It appears that one of the most important factors involved in the regulation of endothelial permeability is vascular endothelial growth factor (VEGF) [9,10]. Evidence indicates that VEGF can disrupt endothelial integrity and increase perme-

Abbreviations: BSA, bovine serum albumin; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; EGCG, epigallocatechin-3-gallate; HMEC-1, human microvascular endothelial cell 1; PCB, polychlorinated biphenyl; PCB 104, 2,2′,4,6,6′-pentachlorobiphenyl; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphatidylinositol 3-kinase; PMA, phorbol-12-myristate-13-acetate; VEGF, vascular endothelial growth factor.

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ability across the endothelial monolayers in vivo or in vitro and promote transendothelial migration of leukocytes and cancer cells, including breast cancer cells [10–12].

Polychlorinated biphenyls (PCBs) are a class of polychlorinated aromatic hydrocarbons composed of 209 discrete congeners. Due to their high lipophilicity and structural stability, PCBs are among the most extensively investigated persistent environmental pollutants that bioaccumulate in the food chain and are concentrated in fatty tissues [13]. In animals and humans, chronic exposure to PCBs produces a variety of effects including neurotoxicity, pro-inflammatory effects, carcinogenesis as well as tumor-promoting effects [14–17]. Indeed, both in vitro and in vivo evidences demonstrated that selected PCBs have carcinogenesis and tumor-promoting activity by inducing oxidative damage in the liver [16,17]. In addition, our group and others have reported that specific PCBs, such as PCB 77, may increase endothelial cell permeability, increase oxidative stress, decrease cellular antioxidants, and activate redox-regulated transcription factors, such as nuclear factor-κB (NF-κB) [15,18].

Among different PCBs, highly *ortho*-chlorinated PCB congeners such as 2,2′,4,6,6′-pentachlorobiphenyl (PCB 104) appear to be of particular interest. For example, this group of PCBs through their estrogenic activity can increase proliferation of breast cancer cells [19]. Our recent study showed that PCB 104 can increase the adhesion of human leukemia cells (THP-1) to human umbilical vein endothelial cells (HUVEC) through upregulation of adhesion molecules [20] as well as induce apoptosis of human microvascular endothelial cells (HMEC) [21]. However, the detailed mechanisms of PCB 104-induced effects on vascular endothelium and transendothelial migration of cancer cells remain unclear.

Because the vascular endothelium plays a regulatory role in transendothelial migration of cancer cells during metastatic process, we hypothesize that *ortho*-substituted non-coplanar PCBs can facilitate transendothelial migration of cancer cells through disruption of vascular endothelial integrity. Results of the present study indicate that PCB 104 can increase endothelial permeability and transendothelial migration of breast cancer cells through the phosphatidylinositol 3-kinase (PI3K)-mediated upregulation of VEGF.

Materials and methods

Reagents

2,2′,4,6,6′-Pentachlorobiphenyl (PCB 104, >99% pure) was purchased from AccuStandard (New Haven, CT) and dissolved in dimethyl sulfoxide (DMSO). Levels of DMSO in the experimental medium were less than 0.1% and did not affect endothelial cell metabolism. LY294002, wortmannin, SU1498, SN50, and SN50M were purchased from Calbiochem (La Jolla, CA). HIF-1α antibody was purchased from Novus Biologicals (Littleton, CO) and antibodies against phospho-Akt (Ser 473) and phospho-c-Jun were obtained from Cell Signaling (Beverly, MS). All other chemicals and reagents including pyrrolidine dithiocarbamate (PDTC) and epigallocatechin-3-gallate (EGCG) were purchased from Sigma (St. Louis, MO).

Cell cultures and PCB treatment

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center, Lexington, KY). HMEC-1 are an immortalized cell line obtained by transformation of human microvascular endothelial cells with the SV40 large T antigen. These cells retain endothelial cell phenotype and functional characteristics [22]. HMEC-1 were cultured in MCDB 131 medium (GibcoBRL; Rockville, MD) enriched with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma), 50 units/ml penicillin, 50 μg/ml streptomycin (GibcoBRL), 1 μg/ml hydrocortisone (Sigma), and 0.01 μg/ml epidermal growth factor (EGF) (Roche; Indianapolis, IN) in 5% CO_2 atmosphere at 37 °C. Before each experiment, the cells were serum-starved in experimental medium containing 1% FBS without EGF for 18 h.

The MDA-MB-231 cells (a metastatic breast cancer cell line) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in suspension in RPMI 1640 medium (GibcoBRL) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Serum concentration of PCBs can reach approximately 3 μM in people exposed to these toxicants [23,24]; however, local levels of PCBs in extracellular space are not known. Therefore, in the present study, cells were treated with a range of PCB 104 concentrations such as 2, 5, 10, or 15 μM. Similar experimental design was used in our previously published manuscripts [20,21]. In a selected experiment, HMEC-1 were pretreated with inhibitors of specific signaling pathways for 30 min before adding PCB 104. The inhibitors were then maintained in the media throughout the PCB 104 exposure. Stock solution of PCB 104 was prepared in DMSO and the same amounts of DMSO as in PCB-treated cells were added to control cultures. Basic composition of experimental medium was the same as that of growth medium, except for serum concentration which was lowered to 1%. In selected experiments, phorbol-12-myristate-13-acetate (PMA; Sigma) at a concentration of 10 nM was used as positive control.

Permeability assay

HMEC-1 (1×10^5 cells) were seeded on fibronectin-coated Transwell polycarbonate filters (12-mm diameter, 0.4-μm pore size, Corning Costar) and allowed to grow to confluence. Fresh complete medium was replaced every 2 days. To confirm confluence of endothelial monolayer, transendothelial electrical resistance (TEER) across the inserts was measured daily using Millicell-ERS voltohm-

meter (Millipore, Bedford, MA). The resistance increased progressively and reached a plateau at day 4 after seeding, indicating the complete formation of the monolayer. Therefore, all experiments were performed 5–6 days after seeding.

To determine its effects on endothelial permeability, PCB 104 with or without pretreatment of inhibitors was added for 24 h to both the lower and the upper compartment of Transwell system. After the cultures were rinsed twice with Kreb-Ringer Glucose (KRG) solution, 1.5 ml of KRG solution was added into the lower chambers of Transwell, and 0.5 ml of FITC-dextran 40 (FD-40, 1 mg/ml in KRG solution) was loaded into the upper chambers. The systems were incubated for 1 h at 37°C in a humidified atmosphere (5% CO_2) and the assay was stopped by removing the upper chambers. Aliquots (0.5 ml) from the lower chambers were transferred to new well of 24-well plate and fluorescence of FD-40 was determined with a microplate spectrofluorometer (Molecular Devices SPECTRA-max GeminiXS) using 483 nm as excitation and 517 nm as emission wavelengths. Relative permeability was expressed by the ratio of FITC-dextran transported into the lower chamber compared to untreated-control group. All assays were performed at least in triplicate.

Cell adhesion assay

Adhesion of MDA-MB-231 cells to HMEC-1 was assessed according to the method of Braut-Boucher et al. [25] with modifications. Briefly, HMEC-1 cells were grown to confluence on fibronectin-coated wells of 24-well plates. The cultures were treated with PCB 104 for 24 h and before the adhesion assay they were washed three times with Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) (HBSS–BSA).

The MDA-MB-231 cells were suspended in the amount of 1.0×10^6 cells/ml HBSS–BSA and labeled with 5 μM calcein-AM (Calbiochem) by 30-min incubation at 37°C followed by three washings with HBSS–BSA. The labeled MDA-MB-231 cells were then incubated with PCB 104-treated HMEC-1 for 30 min at 37°C. Cultures were carefully washed three times with HBSS–BSA to remove nonadherent cells. The adherence was quantified by fluorescence measurements of the attached calcein-labeled MDA-MB-231 cells using excitation of 490 nm and emission of 517 nm. The percentage of MDA-MB-231 cells adherent to the endothelial monolayer was calculated using the formula:

$$\% \text{ adhesion} = (F_a - F_b)/(F_t - F_b) \times 100$$

where F_a is the fluorescence of the adherent MDA-MB-231 cells, F_t is the fluorescence of the total calcein-labeled MDA-MB-231 cells added, and F_b is the background fluorescence of wells. The results are expressed as the percentage of adhesion values determined in control cultures.

Transendothelial cell migration assay

HMEC-1 were seeded in the amount of 5.0×10^4 cells and grown to confluence on fibronectin-coated Transwell polycarbonate filters (6.5-mm diameter, 8.0-μm pore size, Corning Costar). The medium was replaced every 2 days and confluent cultures were exposed to PCB 104 as described for the permeability assay. HMEC-1 were then washed twice with migration medium (serum-free MCDB 131 containing 1% BSA) and the calcein-labeled MDA-MB-231 cells (4.0×10^4 cells) suspended in 100 μl of the same medium were added to the monolayer of HMEC-1 (i.e., to the upper chamber of the Transwell system). After incubation for 10 h, cells were fixed with 4% formaldehyde and washed extensively with PBS. To remove nonmigrating cells, cells on the upper face of the filter were gently scraped using a cotton swab and the migrating tumor cells were observed under fluorescent microscope (Nikon eclipse E600, Nikon, NY). Migrating cells were counted from five random fields using the ×200 magnification. All assays were performed in triplicate.

Conventional and real-time RT-PCR

Total RNA was prepared from PCB 104-treated HMEC-1 using TRI reagent (Sigma) according to the manufacturer's instructions. First strand cDNA was generated from 1 μg of the total RNA using the Reverse Transcription System kit (Promega, Madison, WI) and either oligo dT_{15} primers (for conventional RT-PCR) or random hexamer primers (for real-time RT-PCR). To measure VEGF mRNA expression by conventional RT-PCR, cDNA was amplified by PCR using VEGF-specific primers (sense, 5′-CTA CCT CCA CCA TGC CAA GT-3′, and antisense, 5′-TCT CTC CTA TGT GCT GG CCT-3′, to yield a 311 bp product for VEGF). VEGF-specific primers were designed using the Primer3 software (Whitehead Institute, Cambridge, MA). In addition, β-actin expression was assessed as a housekeeping gene (sense, 5′-AGC ACA ATG AAG ATC AAG AT-3′, antisense, 5′-TGT AAC GCA ACT AAG TCA TA-3′, to yield a 188 bp product) [26]. The PCR reaction mixture consisted of Taq PCR Master Mix (Qiagen, Valencia, CA), 2 μl of cDNA, and 20 pmol of primer pairs in a total volume of 50 μl. PCR was initiated with a hot start (94°C, 5 min) and continued for 25 cycles (VEGF) or 21 cycles (β-actin) of denaturation (94°C, 45 s), annealing (60°C, 45 s), and extension (72°C, 1 min) before the final extension (72°C, 7 min). PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR Gold (Molecular Probes, Eugene, OR) solution for 1 h, and visualized by phosphoimage analysis (FLA-5000, Fuji, Stamford, CT).

VEGF mRNA expression was confirmed by real-time RT-PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR amplification was performed using TaqMan Universal PCR Master Mix

(Applied Biosystems) according to the manufacturer's instructions. Commercially available predeveloped primer pair and TaqMan probe (Applied Biosystems) were used to determine VEGF mRNA levels. PCR cycles consisted of an initial denaturation step at 95°C for 10 min, followed by 95°C for 15 s and 60°C for 60 s (for up to 45 cycles). PCR amplification of 18S RNA (a housekeeping gene) was performed for each sample to normalize VEGF mRNA levels. A standard curve was generated by plotting the threshold cycle (Ct) vs. the log concentration of the serial dilutions of the cDNA from the sample obtained from HMEC-1 treated with 10 μM PCB 104 for 12 h. Each sample was analyzed three times.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as describe earlier [21] with minor modifications. The cells were suspended in 1 ml of lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 0.1% NP-40), lysed for 5 min on ice, and centrifuged at 600 × *g* for 4 min at 4°C to collect nuclei. Then, the nuclear pellets were washed with 1 ml of lysis buffer without NP-40, and then lysed in 50 μl of nuclear extract buffer (20 mM Tris–HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) for 10 min on ice, and centrifuged at 12,000 × *g* for 15 min at 4°C. Nuclear extracts were stored in −80°C freezer before use. Protein concentrations of isolated nuclear extracts were determined using commercial Bradford regent (Sigma). Double-stranded oligonucleotides containing the consensus sequences of the binding sites for transcription factors NF-κB and AP-1 were end-labeled with [γ-^{32}P]-ATP using bacteriophage T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Unincorporated nucleotides were removed by gel filtration chromatography using mini Quick Spin Oligo Columns (Boehringer Manheim Corporation; Indianapolis, IN). Binding reactions were performed with 4 μg (NF-κB) or 2 μg (AP-1) of nuclear protein extracts in a 20 μl volume of reaction mixture (10 mM Tris–Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, and 2 μg of poly[dI-dC]). After adding the reagents, the mixture was incubated for 25 min at room temperature. Then, 40,000 cpm of ^{32}P-labeled specific oligonucleotide probe was added, and the binding mixture was incubated for 25 min at room temperature. In antibody supershift experiments, nuclear protein extracts were incubated for 25 min at room temperature with 2 μg of specific antibody before adding the ^{32}P-labeled specific oligonucleotide probe. Resultant protein–DNA complexes were analyzed on a non-denaturing 5% polyacrylamide gel using 0.25× TBE buffer (50 mM Tris–HCl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4) for 3 h at 150 V. The gel was transferred to Whatman 3MM paper, dried on a gel dryer, and exposed to an X-ray film at −80°C with an intensifying screen.

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined by the 2′,7′-dichlorofluorescein (DCF) method as described earlier [20] with minor modifications. Briefly, confluent HMEC-1 cultured on fibronectin-coated 24-well plates were incubated with PCB 104 for 30 min followed by incubation with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 30 min at 37°C. In selected experiments, cultures were pretreated for 30 min with antioxidants before exposure to PCB 104. At the end of incubation, cultures were rinsed twice with HBSS and 0.5 ml of HBSS was added into each well. The relative fluorescence was assessed using microplate spectrofluorometer (SPECTRA-max GeminiXS, Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths were 485 and 530 nm, respectively. Results were expressed in relative fluorescence units (RFU).

Cell viability assays (MTT assay)

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion assay was performed to assess cell viability. Briefly, confluent HMEC-1 cultures were treated with PCB 104 for up to 48 h. In selected experiments, inhibitors of specific signaling pathways were added 30 min before PCB 104 treatment and then maintained in media throughout the PCB 104 exposure. After incubation, the cells were rinsed two times with PBS and incubated with MTT solution (1 mg/ml in experimental medium) for 4 h at 37°C. The insoluble colored formazan salts were dissolved in DMSO and the absorbance was assessed at 570 nm. Results represent the mean and standard deviation of quadruplicate determinations.

Western blotting

Confluent HMEC-1 seeded onto 100-mm plates, were treated with PCB 104, washed with cold PBS, and lysed with lysis buffer (1% Nonidet P-40, 20 mM Tris–HCl [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 2 mM dithiothreitol, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were incubated for 20 min at 4°C followed by centrifugation at 12,000 × *g* at 4°C. Samples (30 μg of protein/lane) were electrophoresed in 10% SDS-PAGE and transferred onto a Hybond-ECL membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) and incubated overnight at 4°C with anti-phospho-Akt or anti-Akt antibody. The membrane was then washed with TBS-T and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody. After washing three times with TBS, immunoreactive protein bands were visualized with the enhanced chemiluminescence system (Amersham).

Enzyme-linked immunosorbent assay (ELISA)

Medium protein levels of VEGF were quantified by using Quantikine human VEGF immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. This assay recognizes the soluble isoforms VEGF121 and VEGF165. Briefly, HMEC-1 were cultured in fibronectin-coated wells of 6-well plate until confluence. To determine VEGF protein, HMEC-1 were treated with 10 μM PCB 104 for 12 or 36 h. The media were collected, centrifuged, and the supernatants used for ELISA. In addition, HMEC-1 were lysed with 1 N NaOH and protein amounts were determined per well using the Bradford assay (Sigma) to normalize the amounts of VEGF released.

Statistical analysis

Results are expressed as means ± SD. Data were statistically analyzed using one-way ANOVA followed by Stu-

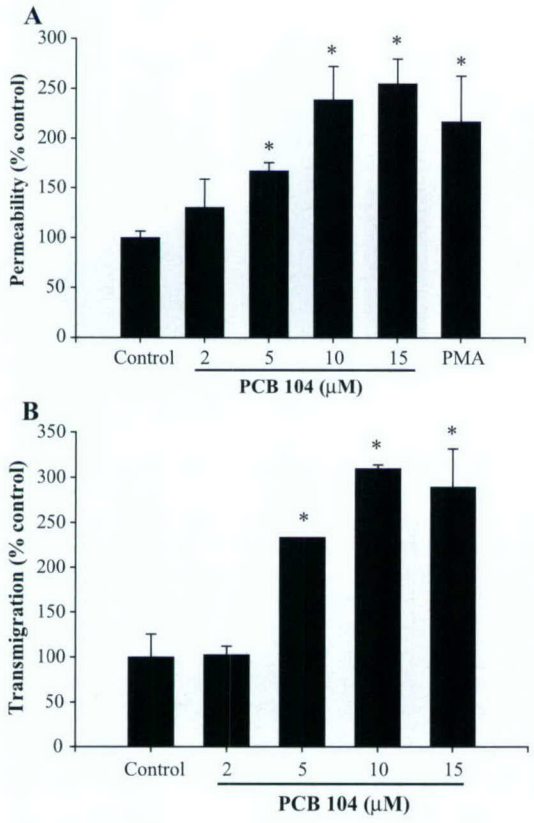

Fig. 1. PCB 104 increases HMEC-1 permeability (A) and transendothelial migration (B) of MDA-MB-231 cells. HMEC-1 were grown to confluence on fibronectin-coated Transwell membranes (0.4-μm pores for permeability and 8-μm pores for transmigration) and exposed to indicated concentrations of PCB 104 for 24 h. Endothelial permeability was measured using FITC-dextran 40 and transmigration was assessed using calcein-labeled MDA-MB-231 cells as described in Materials and methods. Phorbol-12-myristate-13-acetate (PMA) was used as positive control at a concentration of 10 nM. Data are mean ± SD. *Statistically different as compared to control cultures.

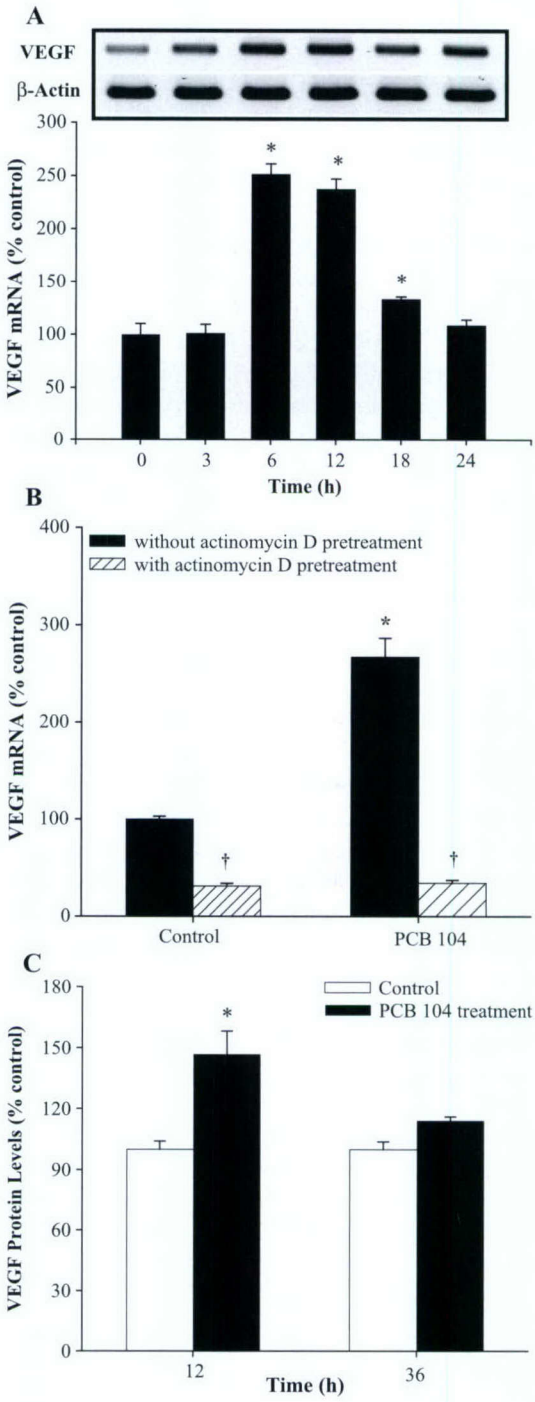

Fig. 2. PCB 104 increases VEGF expression in HMEC-1 at the transcription level. Confluent HMEC-1 cultures were exposed to 10 μM PCB 104 for the indicated time points. VEGF mRNA was determined using conventional RT-PCR (A, upper panel) and real-time RT-PCR (A, lower panel). In addition, HMEC-1 were pretreated for 1 h with 5 μg/ml actinomycin D, an inhibitor of RNA transcription, before exposure to 10 μM of PCB 104 for 8 h. VEGF mRNA was determined using real-time RT-PCR (B). ELISA was employed to assess levels of VEGF protein released to cell culture media (C). Data are mean ± SD. *Statistically different as compared to control cultures. †Values in the cultures pretreated with actinomycin D are statistically different from those in respective controls.

dent's t test. Statistical probability of $P < 0.05$ was considered significant.

Results

PCB 104 increases HMEC-1 permeability and transendothelial migration of MDA-MB-231 cells

The effects of PCB 104 on HMEC-1 permeability were assessed using FITC-dextran 40 (FD-40) and the fibronectin-coated Transwell system with 0.4-μm pore size. Exposure of HMEC-1 to PCB 104 for 24 h resulted in a dose-dependent increase in permeability (Fig. 1A). A prominent disruption of HMEC-1 integrity was observed in cultures treated with 10 μM PCB 104. An increase in PCB 104 concentration to 15 μM did not result in further elevation of FD-40 transfer across HMEC-1 monolayers. In these experiments, 10 nM PMA was used as a positive control.

An increase in endothelial permeability may result in elevated transendothelial migration of cancer cells. Therefore, the effects of PCB 104 on the transmigration of MDA-MB-231 cells across HMEC-1 monolayers were also evaluated in the present study. Calcein-labeled MDA-MB-231 cells and the Transwell system with 8-μm pore size were used in these experiments. To exclude the direct effects of PCB 104 on cancer cells, HMEC-1 cultures were washed twice with migration medium (serum-free MCDB 131 containing 1% BSA) before adding MDA-MB-231 cells. As illustrated in Fig. 1B, PCB 104 treatment induced a dose-dependent increase in the migration of MDA-MB-231 cells across the HMEC-1 monolayers. The effective concentration of PCB 104 was 5 μM and a further increase in PCB 104 level did not potentate these effects.

Because 10 μM PCB 104 effectively increased endothelial permeability and transmigration of tumor cells, this concentration was selected for further experiments which were focused on the mechanisms of PCB 104-induced disruption of endothelial integrity. In addition, treatment with 10 μM PCB 104 for up to 48 h did not show cytotoxic effects as measured by the MTT conversion assay (data not shown).

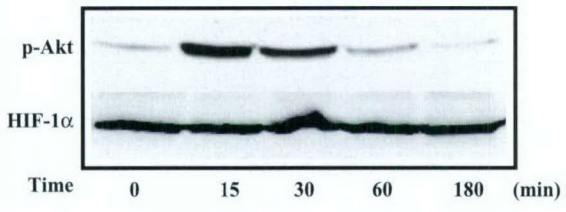

Fig. 3. PCB 104 stimulates PI3K activity in HMEC-1 without activation of HIF-1 transcription factor. Confluent HMEC-1 cultures were treated with 10 μM PCB 104 for the indicated time points. Activation of PI3K was determined by Western blot in whole cell extracts using antibodies against phosphorylated Akt (p-Akt). Levels of HIF-1α were assessed by Western blot in nuclear extracts of control and PCB 104-treated HMEC-1.

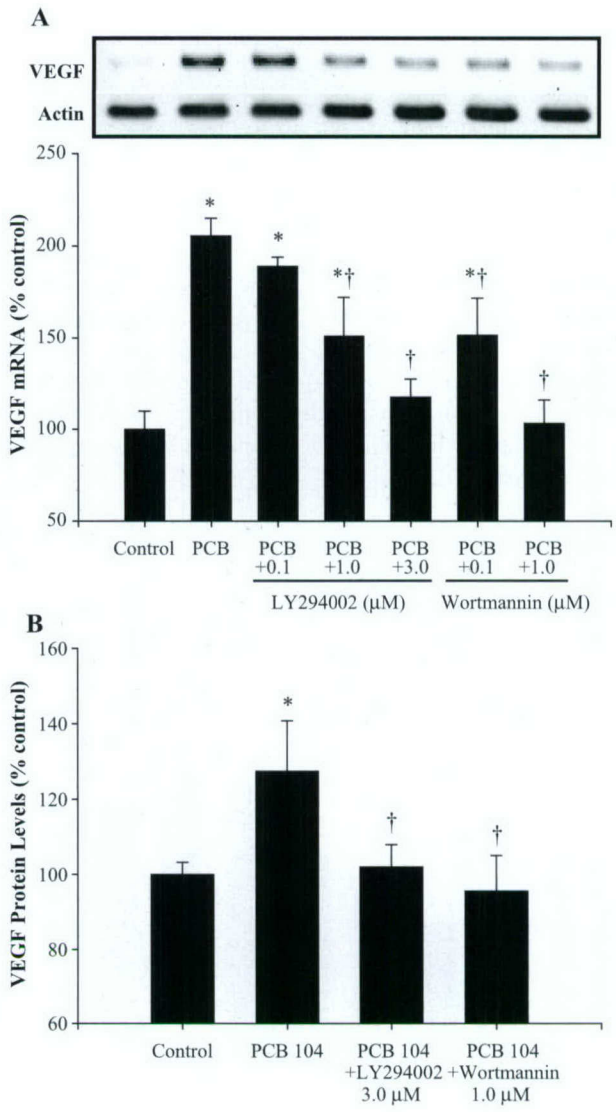

Fig. 4. PI3K mediates PCB 104-induced VEGF expression in HMEC-1. Confluent HMEC-1 cultures were pretreated with the indicated concentrations of LY294002 or wortmannin (inhibitors of PI3K) for 30 min before exposure to 10 μM PCB 104 for 12 h. VEGF mRNA was determined using conventional RT-PCR (A, upper panel) and real-time RT-PCR (A, lower panel). ELISA was employed to assess levels of VEGF protein released to cell culture media (B). Data are mean ± SD. *Statistically different as compared to control cultures. †Levels in the groups PCB 104 plus LY294002 or PCB 104 plus wortmannin are statistically different as compared to those in cultures treated with PCB 104 alone. PCB, PCB 104.

PCB 104 increases VEGF expression at the transcription level through the PI3K-dependent mechanism

VEGF is one of the most important endothelial-derived agents regulating vascular permeability. Therefore, VEGF expression was determined both at the mRNA and protein levels in HMEC-1 exposed to PCB 104 (Fig. 2). As illustrated in Fig. 2A, VEGF mRNA reached maximum levels in HMEC-1 treated with 10 μM PCB 104 for 6 and 12 h and returned to basal values after a 24-h PCB 104 exposure.

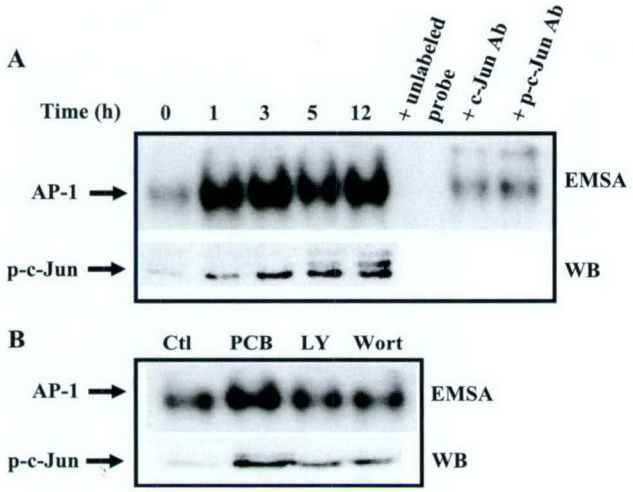

Fig. 5. PCB 104-mediated PI3K activity stimulates AP-1 DNA binding and c-Jun phosphorylation in HMEC-1. Nuclear extracts were isolated from HMEC-1 treated with 10 μM PCB 104 for the indicated time (A). HMEC-1 were pretreated with the PI3K inhibitors LY294002 (3 μM) or wortmannin (1 μM) for 30 min followed by 10 μM PCB 104 for 3 h (B). AP-1 DNA binding activity was analyzed by EMSA. Competition study was performed by the addition of excess unlabeled AP-1 oligonucleotides. Supershift assays were performed with 2 μg of antibodies against c-Jun (c-Jun Ab) or phosphorylated c-Jun (p-c-Jun Ab). In addition, levels of phosphorylated c-Jun (p-c-Jun) were assessed by Western blot (WB). Ctl, control; PCB, PCB 104; LY, LY294002; Wort, wortmannin.

To determine whether PCB 104 induces VEGF mRNA expression at the transcriptional level, confluent HMEC-1's were pretreated for 1 h with actinomycin D, the inhibitor of RNA transcription, and then incubated with PCB 104 for 8 h. PCB 104-mediated VEGF mRNA levels were completely abolished by actinomycin D (Fig. 2B).

PCB 104-mediated increase in VEGF mRNA expression was associated with elevated VEGF protein levels. As shown in Fig. 2C, a 12-h exposure to PCB 104 significantly increased VEGF protein in cell culture media. However, these values returned to control levels in HMEC-1 treated with PCB 104 for 36 h.

Evidence suggests that the phosphatidylinositol 3-kinase (PI3K) pathway may regulate VEGF expression via activation of Akt kinase and stimulation of hypoxia inducible factor-1 (HIF-1) [42,43]. Therefore, the possibility that PCB 104 can stimulate these pathways was also examined in the present study. As shown in Fig. 3, treatment with PCB 104 for 15 or 30 min markedly increased phosphorylation of Akt kinase, the cellular target of PI3K. On the other hand, exposure to PCB 104 did not stimulate HIF-1. As indicated in Fig. 3, a 15- to 180-min treatment with PCB 104 did not

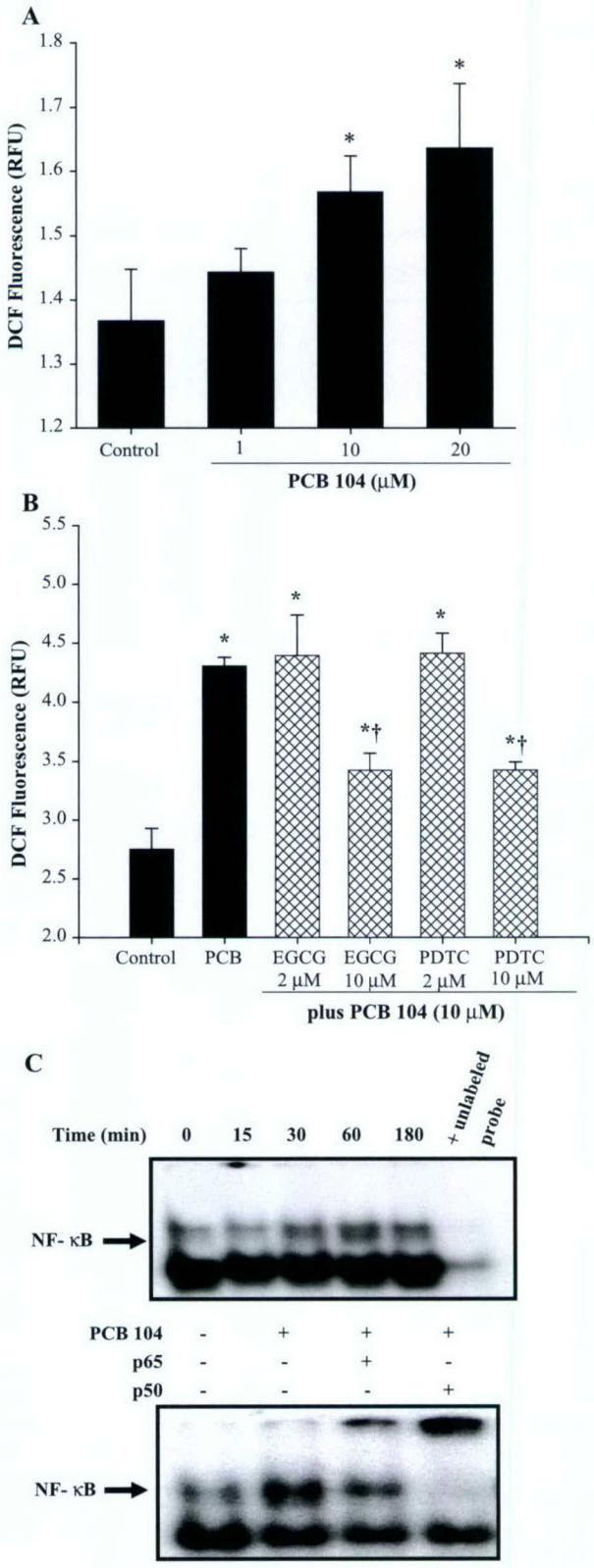

Fig. 6. PCB 104 increases cellular oxidative stress and NF-κB DNA binding activity. DCF fluorescence was measured after 30 min of treatment with the indicated concentrations of PCB 104 (A). To study the effects of antioxidants on PCB 104-mediated oxidative stress, HMEC-1 were pretreated for 30 min with antioxidants PDTC or EGCG at the indicated concentrations (B). NF-κB DNA binding activity was analyzed by EMSA using nuclear extracts prepared from HMEC-1 treated with 10 μM of PCB 104 for the indicated time (C). Competition study was performed by the addition of excess unlabeled NF-κB oligonucleotides. Supershift assay was performed with 2 μg of antibodies against p65 or p50, the components of NF-κB complex. Data are mean ± SD. *Statistically different as compared to control cultures. †Levels in the groups PCB 104 plus EGCG or PCB 104 plus PDTC are statistically different as compared to those in cultures treated with PCB 104 alone. PDTC, pyrrolidine dithiocarbamate; EGCG, epigallocatechin-3-gallate.

affect protein levels of HIF-1α, the major subunit of HIF-1, in nuclear extracts of HMEC-1.

To support the regulatory role of PI3K in PCB 104-induced VEGF mRNA overexpression, pretreatment of HMEC-1 with LY294002 and wortmannin, specific inhibitors of PI3K activation, markedly and in dose-dependent manners decreased PCB 104-mediated stimulation of VEGF mRNA levels as determined by conventional and real-time RT-PCR (Fig. 4A, upper and lower panel, respectively). Consistent with these results, LY294002 and wortmannin also effectively inhibited PCB 104-induced production of VEGF protein (Fig. 4B).

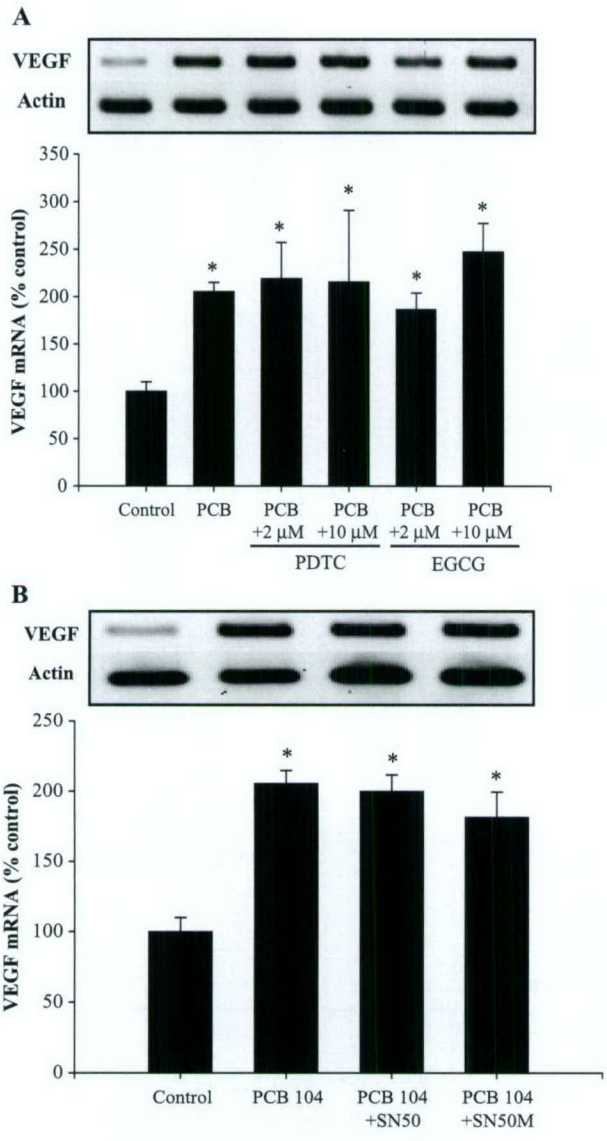

Fig. 7. Oxidative stress-responsive pathways are not involved in PCB 104-induced overexpression of VEGF in HMEC-1. Confluent HMEC-1 cultures were pretreated for 30 min with the indicated concentrations of antioxidants PDTC or EGCG or with 50 μg/ml of SN50 (the inhibitor of NF-κB nuclear translocation) before exposure to 10 μM PCB 104. SN50M is a negative control for the SN50 peptide. VEGF mRNA was determined using conventional RT-PCR (upper panels) and real-time RT-PCR (lower panels) after 12-h incubation of PCB 104. Data are mean ± SD. *Statistically different as compared to control cultures. PCB, PCB 104; PDTC, pyrrolidine dithiocarbamate; EGCG, epigallocatechin-3-gallate.

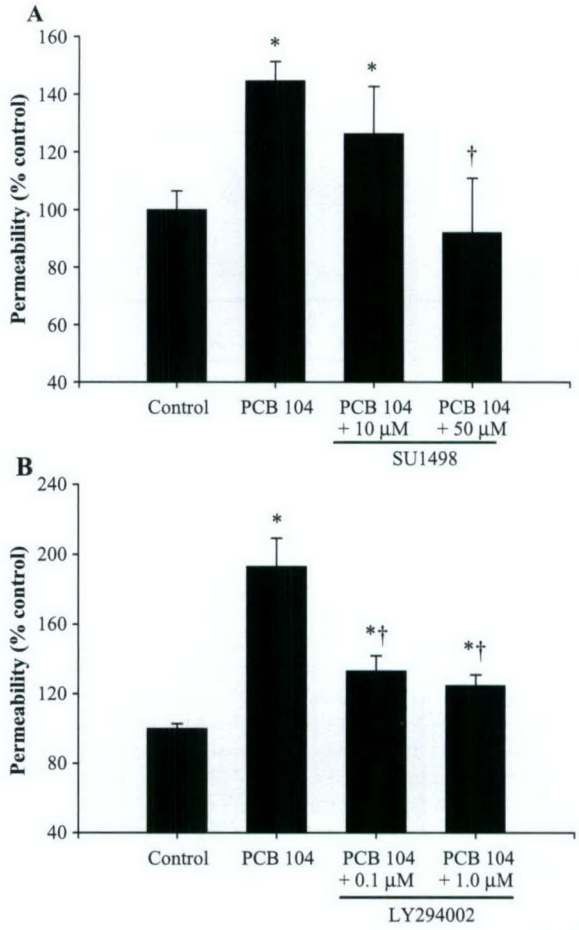

Fig. 8. VEGF and PI3K regulate PCB 104-induced elevation of HMEC-1 permeability. HMEC-1 were grown to confluence on fibronectin-coated Transwell membranes (0.4-μm pores) and pretreated with the indicated concentrations of SU1498 (A) or with LY294002 (B) for 30 min before exposure to 10 μM PCB 104 for 24 h. HMEC-1 permeability was determined as described in Materials and methods. Data are mean ± SD. *Statistically different as compared to control cultures. †Levels in the groups PCB 104 plus SU1498 or PCB 104 plus LY294002 are statistically different as compared to those in the PCB 104 group.

PCB 104-mediated PI3K activation stimulates phosphorylation of c-Jun and AP-1 DNA binding

The human VEGF promoter contains four potential binding sites of AP-1 [47] which play a key regulatory role in VEGF expression [43–46]. Therefore, we assessed AP-1 DNA binding activity and c-Jun phosphorylation in nuclear extracts of HMEC-1 exposed to PCB 104. As illustrated in Fig. 5A, PCB 104 markedly stimulated AP-1 DNA binding. Based on a supershift assay, c-Jun was identified as the major component of PCB 104-induced AP-1 complex. The increase in AP-1 DNA binding was accompanied by elevated levels of phosphorylated c-Jun (Fig. 5A). To illustrate the role of PI3K signaling in these effects, treatment with

LY294002 and wortmannin markedly blocked PCB 104-mediated activation of AP-1 DNA binding and c-Jun phosphorylation (Fig. 5B).

PCB 104-mediated overexpression of VEGF is oxidative stress-independent

The PI3K signaling pathway is known to be regulated by cellular redox status. In addition, PCB congeners were shown to increase intracellular ROS levels in several types of cells and tissues [15,20]. Therefore, a series of experiments was performed to examine the possibility that oxidative stress and nuclear factor-κB (NF-κB) could be involved in PCB 104-mediated VEGF expression. NF-κB is a downstream target of PI3K signaling and a redox-responsive transcription factor.

Exposure of HMEC-1 to PCB 104 induced cellular oxidative stress (Figs. 6A and 6B) and increased DNA-

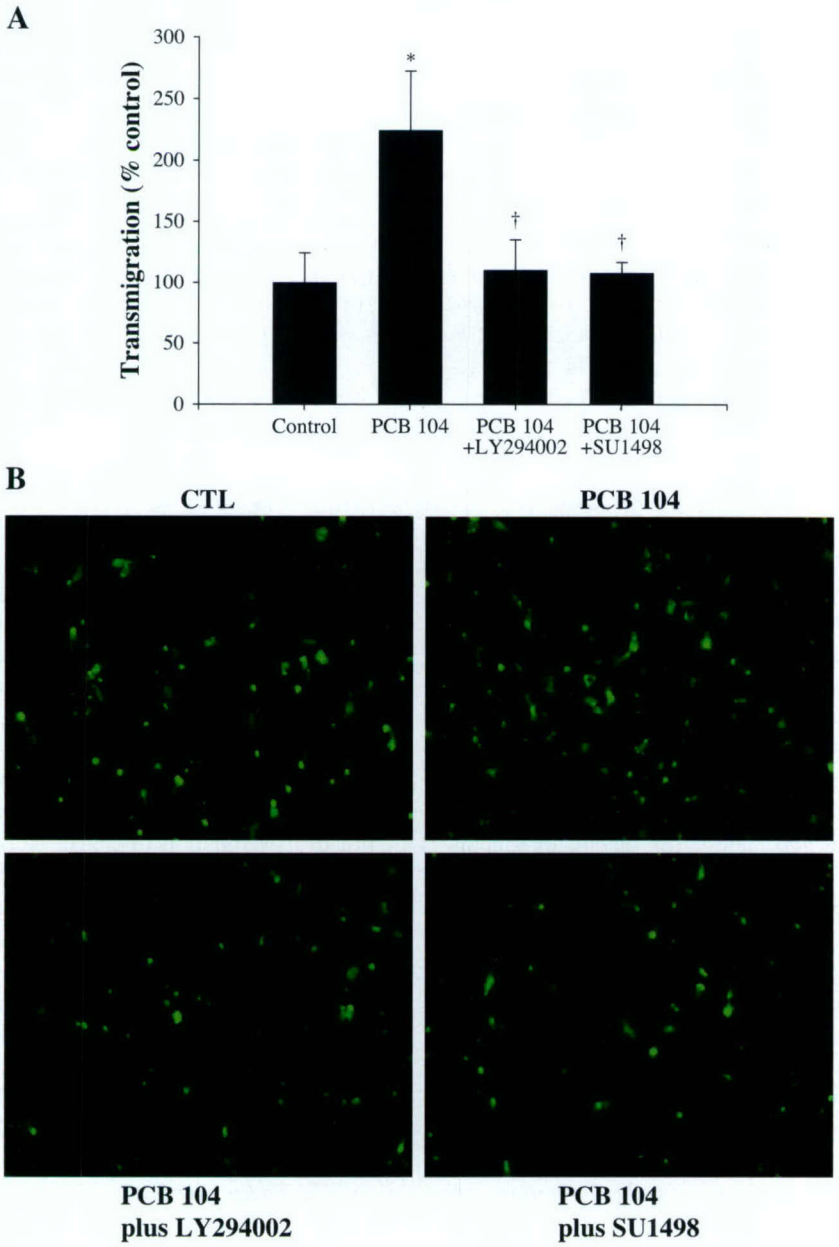

Fig. 9. VEGF and PI3K regulate PCB 104-induced elevation of transendothelial migration of MDA-MB-231 cells across HMEC-1 monolayers. HMEC-1 were grown to confluence on fibronectin-coated Transwell membranes (8-μm pores) and pretreated with LY294002 (1 μM) and SU1498 (50 μM) for 30 min prior to 10 μM PCB 104 and these inhibitors for 24 h. Transmigration of the calcein-labeled MDA-MB-231 cells was quantified (A) as described in Materials and methods. Data are mean ± SD. *Statistically different as compared to control cultures. †Levels in the groups PCB 104 plus LY294002 or PCB 104 plus SU1498 are statistically different as compared to those in cultures treated with PCB 104 alone. (B) Shows the representative fluorescent images of migrating calcein-labeled MDA-MB-231 cells on the lower site of the Transwell membranes. Photomicrographs are representative of comparisons between different experimental groups as described in A. Photomicrographs were taken using a fluorescent microscope with a ×20 objective.

binding activity of NF-κB (Fig. 6C). In addition, pretreatments with antioxidants such as PDTC and EGCG at concentrations of 10 μM significantly attenuated the intensity of cellular oxidation in PCB-104-treated HMEC-1 (Fig. 6B). However, a similar treatment with PDTC and EGCG had no effect on PCB-induced VEGF mRNA expression (Fig. 7A). Moreover, SN50 did not influence VEGF mRNA levels in HMEC-1 treated with 10 μM PCB 104 (Fig. 7B). SN50 contains the nuclear localization sequence of the p50 subunit of NF-κB and it is used as the inhibitor of NF-κB nuclear translocation.

PCB 104-induced alterations of HMEC-1 permeability and transmigration of the MDA-MB-231 cells are regulated by VEGF and PI3K

The majority of cellular effects of VEGF, including an increase in endothelial permeability, are mediated by a high affinity tyrosine kinase receptor known as kinase insert domain (KDR) containing receptor or fetal liver kinase (Flk)-1 (KDR/Flk-1). Therefore, to indicate whether PCB 104-induced overexpression of VEGF can influence alterations in HMEC-1 permeability and transendothelial migration of MDA-MB-231 cells, a series of experiments was performed in which KDR/Flk-1 was functionally blocked with the specific inhibitor SU1498.

As shown in Fig. 8A, pretreatment with SU1498 dramatically and in a dose-dependent manner inhibited PCB 104-induced disruption of HMEC-1 barrier function. Indeed, permeability across HMEC-1 monolayers exposed to 10 μM PCB 104 in the presence of 50 μM SU1498 was in the range of control values. These effects were mimicked by pretreatment with LY294002, the inhibitor of PI3K activity (Fig. 8B). Moreover, both SU1498 and LY294002 significantly blocked an increase in transendothelial migration of MDA-MB-231 cells across HMEC-1 monolayers exposed to PCB 104 (Figs. 9A and 9B). The experimental treatments and conditions employed in these experiments did not affect cell viability as measured by the MTT conversion assay (data not shown).

VEGF is not involved in increased adhesion of MDA-MB-231 cells in PCB 104-treated HMEC-1

We previously reported that PCB 104 can increase adhesion of tumor cells to human endothelial cells. In addition, changes of adhesion properties of the endothelium can modulate the transmigration of cancer cells. Therefore, we examined whether overexpression of VEGF is involved in PCB 104-mediated alterations of adhesion of MDA-MB-231 cells to HMEC-1.

As shown in Fig. 10, treatment with 10 μM PCB 104 for 24 h significantly increased adhesion of MDA-MB-231 cells to the HMEC-1 monolayer. However, pretreatment with LY294002, wortmannin, or SU1498 did not influence these effects, indicating that PCB 104-mediated stimulation of cell adhesion is regulated by distinct mechanisms which do not involve VEGF and PI3K.

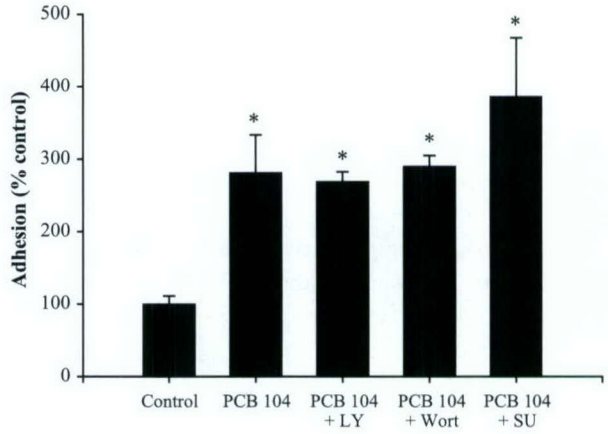

Fig. 10. VEGF and the PI3K pathway are not involved in PCB 104-induced adhesion of MDA-MB-231 cells to HMEC-1. Confluent HMEC-1 cells were pretreated with 3 μM LY294002, 1 μM wortmannin (both PI3K inhibitors), or 50 μM SU1498 (VEGF receptor inhibitor) for 30 min before exposure to 10 μM PCB 104 for 24 h. Adhesion of the calcein-labeled MDA-MB-231 cells was assessed as described in Materials and methods. *Statistically different as compared to control cultures. LY, LY294002; SU, SU1498; Wort, wortmannin.

Discussion

Endothelial cells, which typically form a squamous and quiescent vascular lining, create a selective barrier between the vascular space and the tissues [5,6]. One of the key events in cancer metastasis is transendothelial migration of tumor cells. Indeed, metastasizing tumor cells penetrate through the vascular endothelium and the vessel walls for extravasation and invasion. Thus, the dysfunction of vascular endothelium barrier function may facilitate the widespread dissemination of cancer cells. To support the role of vascular processes in cancer metastasis, it was shown that endothelial injury can promote the localization and metastasis of cancer cells to the lung and that these effects can be attenuated by endothelial repair [2].

Emerging evidence indicates that PCBs may exert profound vascular effects. For example, our group as well as others reported that coplanar PCBs such as PCB 77 and PCB 126, which have dioxin-like structures and are aryl hydrocarbon receptor (AhR) agonist, can increase oxidative stress and induce inflammatory responses in vascular tissue [15,27]. These properties of coplanar PCB may contribute to the disruption of endothelial barrier dysfunction in vitro and in vivo. However, it is not clear if a group of non-coplanar PCB congeners like PCB 104, which are not typical AhR agonists, can disrupt endothelial barrier function and modulate the transendothelial migration of cancer cells. This is an emerging question because recent data demonstrated that specific non-coplanar PCB congeners can exert potent pathological effects such as immunotoxicity, estrogenicity,

and tumor-promoting activity. It was reported that PCB 104 can cause immunosuppression through induction of apoptosis of human monocytic cells [28]. In addition, non-coplanar PCBs, including PCB 104, significantly increased MCF-7 breast cancer cell proliferation; the effect which could be inhibited by co-treatment with an estrogen antagonist hydroxytamoxifen [17]. Recent study from our group demonstrated that PCB 104 upregulated expression of adhesion molecules and increased the adhesion of leukemia cells to human endothelial cells [20]. In addition, results of the present study indicate that PCB 104 can markedly disrupt integrity of the vascular endothelium and increase transmigration of breast tumor cells.

Several vascular mechanisms can be responsible for the facilitation of extravasation and dissemination of tumor cells. For example, upregulation of specific adhesion molecules on the endothelial surface can increase transendothelial migration of cancer cells through the increased adhesion of cancer cells to endothelial cells [1,5]. In addition, transmigration of cancer cells can be augmented by disruption of cell junctions [6,10]. Finally, several soluble factors such as VEGF can induce the disruption of endothelial integrity, which in turn may directly enhance penetration of tumor cells and facilitate establishment of cancer metastasis [9,10]. Microvascular hyperpermeability in proximity to metastatic tumors can also accelerate plasma protein extravasation to stimulate tumor growth [29].

Results of the present study clearly indicate that exposure of HMEC-1 to PCB 104 can increase permeability and transendothelial migration of cancer cells through overexpression of VEGF. Two critical facts support this statement: (1) exposure to PCB 104 increased VEGF expression both at the mRNA and protein levels (Figs. 2A and 2B), and (2) SU1498, the antagonist of the VEGF receptor (KDR/Flk-1), significantly inhibited PCB 104-induced hyperpermeability of HMEC-1 and transendothelial migration of breast cancer cells (Figs. 8 and 9, respectively).

VEGF, also known as vascular permeability factor (VPF), has potent vascular permeable activity specific to vascular endothelial cells implicated in endothelial barrier dysfunction and also plays an essential role in promoting new blood vessel formation (angiogenesis) during tumor development [30,31]. Indeed, inhibition of VEGF function can effectively prevent tumor growth through incomplete blood vessel formation. Thus, VEGF has been recognized as a primary mediator of metastasis formation in a number of human cancers. Although the detailed mechanisms of VEGF-mediated stimulation of hyperpermeability are not known, evidence indicates that the rearrangement and altered expression of endothelial junctional proteins can be involved in this process [12,32,33]. It was also reported that VEGF can induce proliferation and invasion of VEGF receptor-expressing breast cancer cells [34]. Thus, it is possible that VEGF secreted by PCB 104-exposed endothelial cells can affect transendothelial migration of cancer cells both through increased permeability of endothelial monolayers and the alteration of invasive properties of tumor cells.

Recent evidence indicated that VEGF can increase expression of adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) in human endothelial cells. These molecules can increase the adhesiveness and transendothelial migration of tumor cells [35,36]. However, results of the present study indicated that VEGF is not involved in PCB 104-induced elevated adhesiveness of MDA-MB-231 cells to HMEC-1. Indeed, neither functional inhibition of the VEGF receptor nor inhibition of the PI3K signaling did affect adhesion of MDA-MB-231 breast tumor cells to HMEC-1 monolayers (Fig. 10). Thus, PCB 104-induced stimulation of transendothelial migration and adhesion of tumor cells to the vascular endothelium appear to be regulated by distinct mechanisms (Fig. 11). To support this notion, it was reported that tumor cells have higher affinity to extracellular matrix (ECM) components, such as fibronectin, than to the surface of an intact endothelial monolayer [4]. Indeed, a blockage of the adhesion of tumor cells to ECM components significantly prohibited metastatic formation [37].

In the present study, we identified that the PI3K signaling pathway is involved in PCB 104-induced VEGF expression. First, we provided evidence that the PI3K pathway is activated in PCB 104-treated HMEC-1 (Fig. 3); and second, we demonstrated that PI3K inhibitors such as LY294002 or wortmannin can block the effects of PCB 104 on VEGF

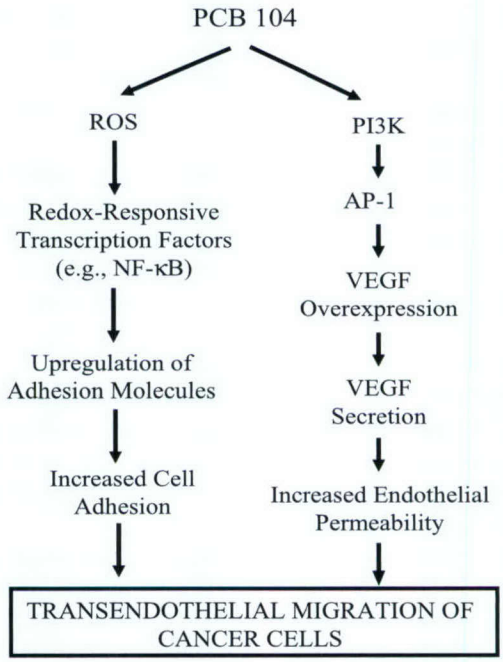

Fig. 11. Proposed diagram of PCB 104-mediated intracellular signaling leading to increased permeability and transendothelial migration of cancer cells. AP-1, activator protein-1; NF-κB, nuclear factor-κB; PCB 104, 2,2′,4,6,6′-pentachlorobiphenyl; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

expression (Fig. 4), endothelial permeability (Fig. 8B), and transmigration of MDA-MB-231 cells (Fig. 9). These findings support recent literature data. For example, it was reported that PI3K plays a role in increased VEGF production induced by several stimuli, including oxidative stress and platelet-derived growth factor [38,39]. In addition, it was shown that expression of constitutively active PI3K or Akt is associated with an increased steady state of VEGF mRNA levels in endothelial cells and fibroblasts [40].

Our data indicate that the induction of VEGF mRNA level by PCB 104 can be completely blocked by pretreatment with actinomycin D, indicating the transcriptional regulation of this process (Fig. 2B). Therefore, to clarify the molecular mechanisms of VEGF mRNA overexpression, we assessed the role of oxidative stress and redox-regulated transcription factors such as HIF-1, AP-1, and NF-κB in PCB 104-induced VEGF expression.

Literature data suggest that reactive oxygen species (ROS) can stimulate VEGF expression in vascular endothelial cells, retina pigment epithelial cells, and keratinocytes [38,41]. We reported that PCB congeners can induce activation of NF-κB, a representative transcription factor of the PI3K pathway, through increased intracellular ROS level [15]. Therefore, in the present study, we tested the possibility that PCB 104-mediated ROS can induce PI3K activation and VEGF expression. Unexpectedly, both antioxidants employed in the present study, PDTC and EGCG, had no effects on PCB 104-induced changes of PI3K and VEGF expression (Fig. 7A). In addition, SN50, the inhibitor of NF-κB nuclear translocation, did not affect VEGF mRNA expression in HMEC-1 exposed to PCB 104 (Fig. 7B). Thus, it appears that PCB 104-induced overexpression of VEGF in HMEC-1 does not require an increase in oxidative stress to induce the PI3K signaling pathway and VEGF expression (Fig. 11).

HIF-1 is a transcription factor which may regulate VEGF gene transcription through the binding to the hypoxia-responsive element (HRE) on the VEGF promoter [42–44]. HIF-1 consists of two subunits, HIF-1α and HIF-1β; however, its transcriptional activity primarily depends on HIF-1α levels translocated into the nucleus [42]. Therefore, we determined HIF-1α protein levels in nuclear extracts isolated from HMEC-1 exposed to PCB 104. As shown in Fig. 3, the nuclear content of HIF-1α was not changed by PCB 104 exposure, indicating that the HIF-1 signaling pathway is not involved in PCB 104-induced VEGF expression in HMEC-1.

The promoter of the human VEGF gene contains four potential AP-1 binding sites [47]. Moreover, AP-1, and especially its subunit c-Jun, can regulate VEGF gene transcription with or without functional cooperation with HIF-1 [43–46]. For example, insulin-like growth factor 1 (IGF-1) can stimulate VEGF synthesis through the Akt-dependent pathway via activation of AP-1 and HIF-1α [44]. In contrast, lead-mediated induction of VEGF expression in human astrocytes is regulated by the AP-1-dependent signaling pathway, independent of HIF-1 activation [45]. Similarly, our data indicate that PCB 104 can enhance phosphorylation of c-Jun and AP-1 DNA binding activity through the PI3K pathways (Fig. 5) without affecting HIF-1α protein levels (Fig. 3). These findings suggest that PCB 104 may stimulate VEGF gene transcription in HMEC-1 through the PI3K–Akt pathway via AP-1 activation and independently of HIF-1 (Fig. 11).

In conclusion, the present study indicates that exposure of HMEC-1 to PCB 104 can lead to increased endothelial permeability and transendothelial migration of breast tumor cells. It appears that overexpression of VEGF via the PI3K-mediated mechanism can be the underlying mechanisms of these effects (Fig. 11). These results suggest that highly *ortho*-substituted non-coplanar PCB congeners such as PCB 104 can induce vascular alterations that may promote the development of blood-borne metastases.

Acknowledgments

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2,2′,4,6,6′-Pentachlorobiphenyl (PCB 104) induces apoptosis of human microvascular endothelial cells through the caspase-dependent activation of CREB

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Abstract

It has been proposed that endothelial integrity can play an active regulatory role in the extravasation of tumor cells during cancer metastasis. Since polychlorinated biphenyls (PCBs) have been shown to cause endothelial cell activation or injury and to lead to various diseases that involve dysfunction of the vascular endothelium, the present study was designed to determine the cellular and molecular signaling mechanisms of PCB-induced apoptosis in human microvascular endothelial cells (HMEC-1). A significant and marked decrease in cell viability was observed in HMEC-1 treated with 2,2′,4,6,6′-pentachlorobiphenyl (PCB 104) in a time- and dose-dependent manner. Exposure of HMEC-1 to PCB 104 also dramatically induced internucleosomal DNA fragmentation. However, the caspase inhibitor zVAD-fmk significantly reversed the PCB 104-induced DNA fragmentation in HMEC-1, suggesting that endothelial cell death induced by PCB 104 exposure is, at least in part, due to caspase-dependent apoptotic pathways. To elucidate the molecular signaling mechanisms of PCB 104-induced apoptotic cell death in human microvascular endothelial cells, the present study focused on the effects of acute exposure of PCB 104 on the activation of several transcription factors, such as cAMP responsive element-binding protein (CREB), activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and signal transducers and activators of transcription (STAT1), which have been known to play a pivotal role in the molecular signaling cascades for the induction of apoptosis. A series of electrophoretic mobility shift assay showed that PCB 104 specifically increased only CREB DNA-binding activity in a dose-dependent manner. AP-1, NF-κB, and STAT1, however, were not activated. In addition, zVAD-fmk significantly and dose-dependently blocked the CREB activation enhanced by PCB 104 exposure. These results suggest that PCB-induced death of human microvascular endothelial cells is mediated, at least in part, via the caspase-dependent apoptotic pathways and that the selective activation of CREB is involved in this process.

Keywords: PCBs; Endothelial cells; Apoptosis; Caspases; CREB; Metastasis

Introduction

Hematogenous metastasis is a complex process that requires multiple steps. It has been proposed that the extravasation of tumor cells from the circulation to extravascular tissues is one of the most critical events in this process. The extravasation process allows the circulating tumor cells to pass through endothelial barriers to reach selective metastatic sites (Saiki, 1997). Evidence indicates that this step in the metastatic cascade can be regulated by the microvasculature environment, which can influence the integrity of the vascular endothelium. For example, it has been shown that endothelial damage induced by oxidative stress promotes the localization and metastasis of circulating cancer cells to the lung in an animal model of pulmonary microvascular

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injury and that this effect is attenuated after endothelial repair (Lafrenie et al., 1992). It was also demonstrated that free radical-mediated endothelial cell damage can facilitate the metastasis of pancreatic tumor cells (Shaughnessy et al., 1991). In addition, several soluble factors derived from tumor cells during their interactions with endothelial cells can induce endothelial cell retraction, which in turn may play a crucial role in the establishment of cancer metastasis (Honn et al., 1994; Nakamori et al., 1997). We have also shown that treatment of human microvascular endothelial cells with linoleic acid can induce endothelial dysfunction, manifested by overexpression of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1, processes that can contribute to tumor cell metastasis (Lee et al., 2001; Park et al., 2002). Furthermore, Kebers et al. (1998) have demonstrated that cells derived from solid tumors can impair endothelial integrity by inducing endothelial cell apoptosis. These in vivo and in vitro studies have provided strong evidence that the integrity of the vascular endothelium plays an active regulatory role in cancer metastasis.

Polychlorinated biphenyls (PCBs) are a class of polychlorinated aromatic hydrocarbons composed of 209 discrete congeners. Because of high lipophilicity as well as high stability of these compounds, PCBs are considered serious global environmental pollutants (Kimbrough, 1995; Kodavanti et al., 1995). A compelling body of in vivo and in vitro studies has proven that exposure to PCBs can cause extensive toxic effects, including neurotoxicity, hepatotoxicity, carcinogenicity, immunotoxicity, and cardiotoxicity (Hwang et al., 2001; Jo et al., 2001; Kimbrough, 1995; Kodavanti et al., 1995; Silberhorn et al., 1990; Twaroski et al., 2001; Yoo et al., 1997). It has been recently reported that apoptosis may contribute to PCB-induced toxicity. For example, it was demonstrated that apoptotic cell death induced by 2,2′,4,6,6′-pentachlorobiphenyl (PCB 104) or Aroclor 1254 (a commercially produced PCB mixture) may be one possible mechanism of PCB-mediated immunosuppression of human monocytic cells or murine splenocytes (Shin et al., 2000; Yoo et al., 1997). In addition, Hwang et al. (2001) indicated that 2,2′,5,5′-tetrachlorobiphenyl (PCB 52) can induce apoptosis of neuronal SK-N-MC cells in a process that involves down-regulation of Bcl-2 expression. We have also reported that exposure to 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) can stimulate apoptotic death of endothelial cells via antioxidant-sensitive mechanisms (Slim et al., 2000).

It has been hypothesized that PCBs can cause vascular endothelial cell activation or injury and can thus be implicated in the diseases that involve dysfunction of the vascular endothelium (Hennig et al., 2001). To support this hypothesis, we demonstrated that certain PCBs may play a role in the development of atherosclerosis by causing endothelial cell dysfunction, induction of oxidative stress, and depletion of cellular glutathione status (Hennig et al., 2001; Slim et al., 2000; Toborek et al., 1995). Our recent studies also have shown that selected dietary lipids may further increase the toxic effects of PCBs by cross-amplifying mechanisms, leading to dysfunction of the vascular endothelium (Hennig et al., 1999; Slim et al., 2001).

Although a growing body of evidence has indicated that PCBs can significantly contribute to endothelial cell dysfunction in vivo and in vitro, little information is currently available on detailed mechanisms underlying PCBs-mediated toxic vascular effects. Therefore, the aim of the present study was to investigate the cellular and molecular signaling mechanisms of PCB-mediated endothelial cell apoptosis. This study was performed using PCB 104, a typical representative of *ortho*-substituted, noncoplanar PCB congeners. We demonstrate that PCB 104 specifically activates cAMP-responsive element-binding protein (CREB), leading to the induction of apoptotic death of human microvascular endothelial cells.

Materials and methods

Endothelial cell culture and reagents. Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center). Because tumor cell extravasation occurs generally within the microvasculature (Alby and Auerbach, 1984), HMEC-1 provide the best physiological cell model in research related to vascular metastasis. HMEC-1 were cultured in MCDB 131 medium (GibcoBRL, Rockville, MD) enriched with 10% fetal bovine serum, 2 mM L-glutamine (Sigma, St. Louis, MO), 1% antibiotic-antimycotic (GibcoBRL), 1 μg/ml hydrocortisone (Sigma), and 0.01 μg/ml epidermal growth factor (Roche, Indianapolis, IN) in a 5% CO_2 atmosphere at 37°C. PCB 104 (>99% pure) was purchased from AccuStandard (New Haven, CT) and dissolved in DMSO. The level of DMSO in the experimental medium was 0.1%, i.e., the concentration that does not affect endothelial cell metabolism. z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Calbiochem (La Jolla, CA). Serum concentration in the experimental medium was 5%.

Viability assay. Cell viability was determined with the standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) conversion assay (Mattson et al., 1995). Briefly, HMEC-1 were exposed to increasing concentrations of PCB 104 (1.0, 10, and 20 μM) for 24, 48, or 72 h. Following treatment, experimental media were removed, and cultures were incubated at 37°C for 4 h with MCDB 131 medium containing 0.5 mg/ml MTT (Sigma). At the end of the incubation period, the medium was aspirated and the formazan product was solubilized with DMSO. Absorbance at 570 nm was measured for each well using a SPECTRAmax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Table 1
DNA oligonucleotides used for electrophoretic mobility shift assay

Probe	Sequence[a]
CREB	AGAGATTGCCTGACGTCAGAGAGCTAG
AP-1	CGCTTGATGAGTCAGCCGGAA
NF-κB	AGTTGAGGGGACTTTCCCAGGC
STAT1	ATTCGATCGGGGCGGGGCGAGC

[a] All sequences are from 5′ to 3′; only the sense strand is shown.

DNA ladder assay. DNA ladder assay was performed as described previously (Lee et al., 2000). Cells were collected by trypsinization and the cell pellets were suspended in 100 μl of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% NP-40 for 10 s at room temperature. After centrifugation at 850*g* for 5 min, supernatants were collected and treated with 1% SDS and RNase A (5 mg/ml) for 2 h at 56°C, followed by proteinase K (2.5 mg/ml) for 2 h at 37°C. The samples were mixed with 0.5 vol of 10 M ammonium acetate and 2.5 vol of ice-cold ethanol and incubated for 1 h at −80°C. The DNA was precipitated by centrifugation at 13,400*g* for 20 min, washed with 80% ice-cold ethanol, air dried, and resuspended in 50 μl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). The concentration of DNA was measured by a spectrophotometer, and 4 μg of cellular DNA was fractionated by electrophoresis on a 2% agarose gel. After staining with SYBR Gold (Molecular Probes, Eugene, OR) for 1 h, the internucleosomal DNA cleavage (DNA laddering) was visualized using phosphoimaging technology (Fuji FLA-2000, Stamford, CN).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to the method of Beg et al. (1993) with minor modification (Toborek et al., 2002). The cells were suspended in 1 ml of lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and 0.1% NP-40), lysed for 5 min on ice, and centrifuged at 600*g* for 4 min at 4°C to collect nuclei. The nuclear pellets were then washed with 1 ml of lysis buffer without NP-40, lysed in 100 μl of nuclear extract buffer (20 mM Tris–HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol) for 10 min on ice, and centrifuged at 18,300*g* for 15 min at 4°C. Supernatants that contain nuclear extracts were frozen immediately in liquid nitrogen and transferred to a −80°C freezer before using. Protein concentrations of isolated nuclear extracts were determined as described by Bradford (1976).

Individual double-stranded oligonucleotides containing the consensus sequences of the binding sites for transcription factors CREB, activator protein-1 (AP-1), nuclear factor-κB (NF-κB), or signal transducers and activators of transcription (STAT1) (Table 1) were end labeled with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase. The reaction mixture consisted of 70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1.75 pmol of double-stranded oligonucleotides, 30 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ), and 20 units of T4 polynucleotide kinase (Promega, Madison, WI) in a total volume of 20 μl. The reaction was incubated for 1 h at 37°C. Following incubation, T4 polynucleotide kinase was inactivated by placing the tube for 10 min at 68°C on a heat block. Unincorporated nucleotides were removed by gel filtration chromatography using mini Quick Spin Oligo Columns (Boehringer Manheim Corporation, Indianapolis, IN). Binding reactions were performed in a 20-μl volume containing 2 μg of nuclear protein extracts (1 μg to analyze AP-1-binding activity), 10 mM Tris–Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, and 2 μg of poly[dI-dC], which was used as a nonspecific competitor. After adding the reagents, the mixture was incubated for 25 min at room temperature. ^{32}P-labeled specific oligonucleotide probe (40,000 cpm) was then added, and the binding mixture was incubated for 25 min at room temperature. For supershift experiments, nuclear protein extracts were incubated for 25 min at room temperature with 4 μg of specific antibody against components of the CREB complex, such as CREB-1 or ATF-1, before adding the ^{32}P-labeled specific oligonucleotide probe. Mouse monoclonal anti-CREB-1 and mouse monoclonal anti-ATF-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Resultant protein–DNA complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel (7.5 ml of distilled water, 10 ml of 0.5× TBE buffer, 2.5 ml of 40% acrylamide/bis solution, 0.2 ml of 10% ammonium persulfate, and 0.02 ml of TEMED) using 0.25× TBE buffer (50 mM Tris–Cl, 45 mM boric acid, and 0.5 mM EDTA, pH 8.4) for 3 h at 150 V. The gel was transferred to Whatman 3MM paper, dried on a gel dryer, and exposed to a X-ray film at −80°C with an intensifying screen. The intensity of the bands corresponding to specific transcription factor binding was determined using UN-SCAN-IT gel image analysis software (Silk Scientific, Orem, UT). The values of relative pixel intensity were given below each image.

Statistical analysis. Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS, Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment means were compared using the Bonferroni least significant difference procedure. Statistical probability of $p < 0.05$ was considered significant.

Results

PCB 104 induces microvascular endothelial cell apoptosis

Fig. 1 depicts the effects of PCB 104 on viability of HMEC-1, as measured by the MTT conversion assay. Com-

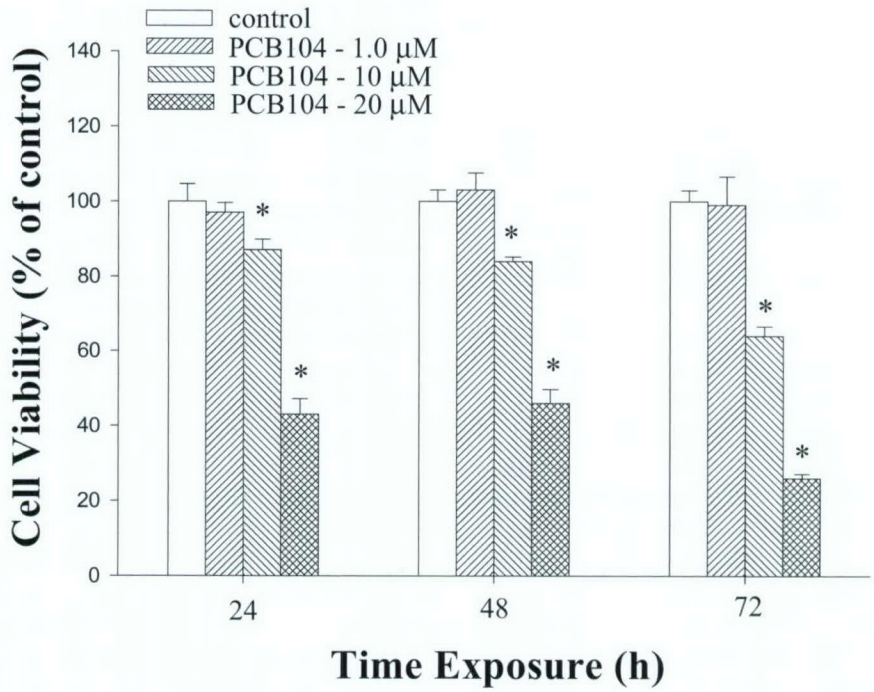

Fig. 1. PCB 104 induces a decrease in viability of HMEC-1. Cells were incubated with the indicated amounts of PCB 104 for up to 72 h, and cell viability was measured by the MTT conversion assay. Cell viability was expressed as the percentage of untreated control cell cultures. Data shown are the means ± SD of six determinations. *Statistically significant compared to the control group ($p < 0.05$).

pared to control cultures, exposure of HMEC-1 to PCB 104 at doses of 10 and 20 μM caused both time- and dose-dependent cytotoxic effects. However, cytotoxicity was not observed in HMEC-1 cultures treated with a low concentration of PCB 104 (1.0 μM) for up to 72 h.

Apoptosis has been characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or their multiples. These fragments are readily detected as a DNA ladder by agarose gel electrophoresis, and in fact such a ladder pattern has been regarded as the most characteristic hallmark of apoptosis (Kubasiak et al., 2002; Nagata, 2000; Ray et al., 2001). Therefore, DNA ladder assays were performed in HMEC-1 cultures exposed to PCB 104 to determine whether PCB 104 can induce endothelial cell apoptosis. As illustrated in Fig. 2A, treatment of HMEC-1 with 20 μM PCB 104 dramatically, and in a time-dependent manner, induced internucleosomal DNA fragmentation. Significant DNA laddering was detected as early as 16 h after treatment with 20 μM PCB 104. In addition, effects of different concentrations of PCB 104 on DNA fragmentation were examined (Fig. 2B). Consistent with the cell viability assay illustrated in Fig. 1, DNA laddering was not detected in HMEC-1 treated with PCB 104 at the concentration of 1.0 μM. However, the internucleosomal DNA fragmentation was significantly induced in HMEC-1 exposed to 10 or 20 μM PCB 104 for 24 h. Longer exposure times resulted in internucleosomal DNA fragmentations also in control cell cultures. Therefore, DNA ladder assays were restricted to a maximum 24 h of PCB 104 exposure.

PCB 104 triggers caspase-dependent apoptotic pathway in human microvascular endothelial cells

Activation of caspases is a common pathway leading to execution of apoptotic cell death. Therefore, to study a

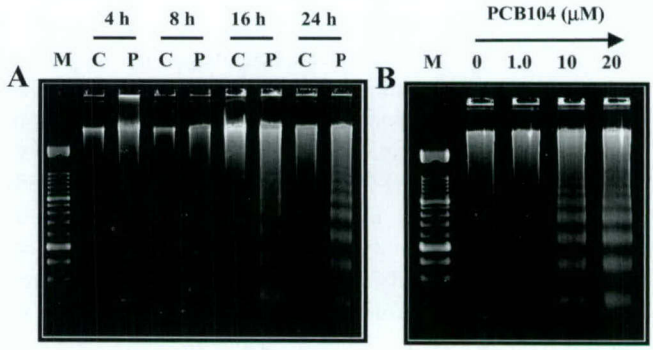

Fig. 2. (A) Time-dependent induction of DNA fragmentation in HMEC-1 by PCB 104. Cells were incubated with 20 μM PCB 104 for up to 24 h. Following treatment exposures, DNA was extracted, fractionated by 2% agarose gel electrophoresis, and visualized using phosphoimaging technology. M, molecular weight markers (100-bp DNA ladder); C, control cells; P, cells exposed to 20 μM PCB 104. (B) Dose-dependent induction of DNA fragmentation in HMEC-1 by PCB 104. Cells were incubated with increasing concentrations of PCB 104 (1.0, 10, or 20 μM) for 24 h and analysis was performed as described in A.

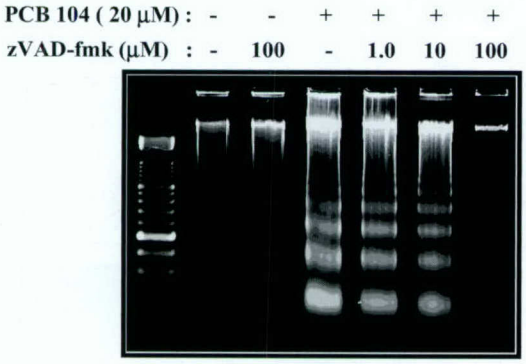

Fig. 3. Caspase inhibitor zVAD-fmk prevents the PCB 104-induced DNA fragmentation in HMEC-1. Cells were pretreated with the indicated concentrations of zVAD-fmk (1.0, 10, and 100 μM) for 2 h and then exposed to PCB 104 (20 μM) for 24 h. Following treatment exposures, DNA was extracted, fractionated by 2% agarose gel electrophoresis, and visualized using phosphoimaging technology.

possible involvement of the caspase pathway in PCB 104-induced apoptosis, DNA laddering was analyzed in HMEC-1 cultures treated with PCB 104 in the presence of a general caspase inhibitor, zVAD-fmk. As illustrated in Fig. 3, zVAD-fmk alone did not show any effect on DNA fragmentation in HMEC-1 compared to nontreated control cultures. However, zVAD-fmk at the concentration of 100 μM completely prevented the ability of PCB 104 to induce the internucleosomal DNA fragmentation in HMEC-1. These data indicate that PCB 104 activates a caspase-mediated apoptotic pathway in human microvascular endothelial cells.

PCB 104 specifically activates CREB DNA-binding activity in human microvascular endothelial cells

To investigate the molecular signaling pathways of endothelial cell apoptosis induced by PCB 104, a series of EMSA was performed. Table 1 depicts sequences of oligonucleotide probes that were employed in these studies. Fig. 4A illustrates the effects of PCB 104 exposure on the DNA-binding activity of CREB transcription factor in HMEC-1. A low level of CREB DNA-binding activity was observed in nuclear extracts isolated from control cultures or HMEC-1 treated with 1.0 μM PCB 104. In contrast, exposure of HMEC-1 to both 10 and 20 μM PCB 104 markedly enhanced CREB DNA-binding activity. The magnitude of these changes was determined by densitometric analysis, and the values of the combined intensity of the upper and lower CREB bands are presented in Fig. 4A. Specificity of the CREB DNA-binding was determined using two different experimental approaches, namely molar excess of unlabeled oligonucleotide containing the consensus CREB binding site and specific antibody against CREB-1 or ATF-1 proteins. As shown in Fig. 4A (left, last lane), molar excess of competitor oligonucleotide completely diminished the bands that corresponded to CREB DNA-binding. In addition, incubation of nuclear extracts with anti-CREB-1 antibody markedly diminished both the upper and the lower CREB band (Fig. 4A, right). In contrast, when the samples were incubated with anti-ATF-1 antibody, only the upper band disappeared. These results indicate that the lower band can be CREB-1/CREB-1 homodimer and the upper band most likely is ATF-1/CREB-1 heterodimer. However, without further experiments using specific antibodies against other members of CREB family, we cannot exclude that other CREB components also can be involved in this binding activity.

Several other transcription factors, such as AP-1, NF-κB, or STAT1 can be regulated by cellular redox status and be involved in apoptotic cell death. Therefore, EMSA studies were also performed to determine effects of PCB 104 on DNA-binding activities of these transcription factors. In addition, tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) were used in these experiments as positive controls to illustrate specificity of analysis and treatment-dependent activation of individual transcription factors. As indicated in Fig. 4B, treatment of HMEC-1 with PCB 104 for 1 h did not activate AP-1, NF-κB, or STAT1. Thus, it appears that CREB transcription factor is specifically activated by PCB 104.

Activation of CREB may be involved in the signaling pathways of PCB 104-induced apoptosis of HMEC-1

To investigate a possible involvement of CREB activation in caspase-dependent signaling cascades of PCB 104-induced apoptosis, CREB DNA-binding activity was studied in HMEC-1 treated with PCB 104 in the presence or absence of zVAD-fmk. As illustrated in Fig. 5, zVAD-fmk markedly and in a dose-dependent manner attenuated CREB DNA-binding activity induced by PCB 104 treatment. As revealed by densitometric analysis of the combined intensity of the upper and lower CREB bands, CREB-binding activity in the nuclear extracts isolated from HMEC-1 treated with PCB 104 in the presence of 100 μM of zVAD-fmk was in the range of control values.

Discussion

The vascular endothelium forms an interface between the blood and underlying layers of vessel walls, and thus it is exposed to a variety of pathophysiologic stimuli, such as environmental toxins, including PCBs. It was suggested that endothelial cells may be targets for bioactivation and toxicity of environmental toxins, including PCBs (Annas et al., 1998). However, only sparse information is available about the effects of PCBs on endothelial cell metabolism. Our group (Hennig et al., 1999; Slim et al., 2001; Toborek et al., 1995) showed that exposure of endothelial cells to selected PCBs can result in the induction of cellular oxidative stress, decrease in cellular antioxidants, and production of inflam-

matory cytokines, such as intereukin-6. PCBs were also demonstrated to induce vascular changes in other species. For example, exposure to PCBs produced vascular lesions in the placental labyrinthine zones of viable fetuses in minks, induced degeneration of endothelial cells, and formed thrombi and hemorrhages (Backlin et al., 1998). The presence of extracellular fluid between the interstitial layer of maternal vessels and the syncytiotrophoblast also indicated compromised endothelial integrity (Backlin et al., 1998). We hypothesized that PCB-mediated alterations of endothelial cell metabolism can play a role in the development of atherosclerosis and/or the formation of blood-borne metastasis (Hennig et al., 2002). It appears that PCB-induced endothelial cell apoptosis can contribute to these pathological processes.

Evidence indicates that the toxicological effects of PCBs can be associated with cytotoxicity that occurs either via apoptosis or necrotic mechanisms in a variety of cell types (Hwang et al., 2001; Inglefield et al., 2001; Shin et al., 2000; Slim et al., 2000; Yoo et al., 1997). In agreement with previous studies, exposure of HMEC-1 to PCB 104 resulted in a significant decrease in cell viability and induced cellular apoptosis, an effect that was prevented by treatment with zVAD-fmk, a general caspase inhibitor. Thus, it appears that the caspase pathway is critically involved in HMEC-1 apoptotic death induced by PCB 104. Caspases have been recognized to play a major role in the execution of apoptosis induced by a variety of stimuli (Nicholson and Thornberry, 1997). Caspases are able to cleave a wide range of substrates, including those directly involved in the execution of the apoptotic death program. They are normally present as inactive zymogens in the cellular cytoplasm, and their activation during cell apoptosis is achieved hierarchically, so that activation of the upstream members of this family initiates the caspase cascade by cleavage and subsequent activation of downstream caspases (Salvesen and Dixit, 1999).

Several redox-responsive transcription factors, such as CREB, AP-1, NF-κB, STAT1, can play a pivotal role in the molecular signaling cascades involved in apoptotic cell death (Jean et al., 1998; Pentikainen et al., 2002; Reusch and Klemm, 2002; Riccio et al., 1999; Saldeen et al., 2001; Sanceau et al., 2000; Somers et al., 1999; Welsh, 1996; Zhao et al., 1997). Therefore, DNA-binding activities of these transcription factors were determined in the present study to elucidate the molecular signaling mechanisms of PCB 104-induced apoptotic cell death in human microvascular endothelial cells. Surprisingly, exposure to PCB 104 did not activate AP-1, NF-κB, or STAT1 in HMEC-1 (Fig. 4B). In fact, among all transcription factors analyzed in the present study, PCB 104 selectively induced CREB DNA-binding activity. These data are in agreement with the recent studies by Inglefield et al. (2001), which demonstrated that acute exposure of rat cortical neuronal cells to the PCB mixture Aroclor 1254 (A1254) can induce CREB phosphorylation.

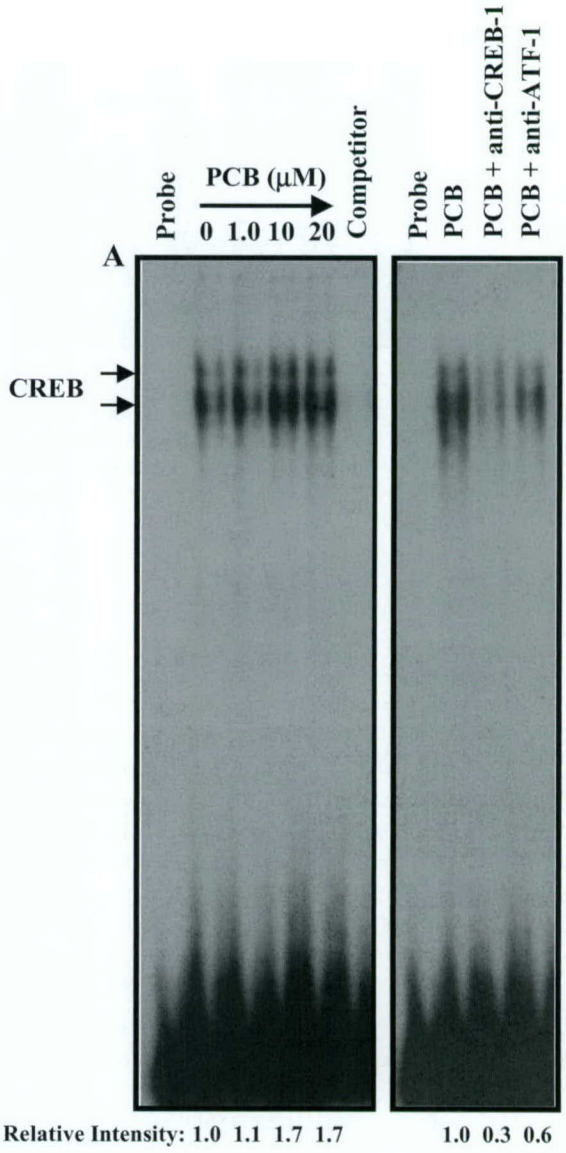

Fig. 4. (A) (Left) PCB 104 enhances CREB DNA-binding activity in HMEC-1. Cells were either untreated or treated with the indicated amounts of PCB 104 (1.0, 10, or 20 μM) for 1 h. Nuclear extracts were prepared and analyzed by EMSA. Competition study was performed by the addition of excess unlabeled CREB DNA-binding consensus sequence using nuclear extracts prepared from HMEC-1 treated with 20 μM PCB 104. (Right) Supershift analysis of CREB DNA-binding activity. Nuclear extracts were prepared from cells treated with 20 μM PCB 104 for 1 h and incubated with anti-CREB-1 or anti-ATF-1 antibody for 25 min before the addition of ^{32}P-labeled probe. (B) Exposure to PCB 104 does not activate AP-1, NF-κB, or STAT1 in HMEC-1. Treatments and analyses were performed as described in A.

The CREB family of transcription factors belongs to the leucine zipper class of proteins involved in activating genes through binding to the cAMP- responsive element (CRE) palindromic octanucleotide, TGANNTCA. A variety of protein kinases, including protein kinase A, mitogen-activated protein kinases (MAPKs), and Ca^{2+}/calmodulin-dependent protein kinases have been reported to phosphorylate CREB

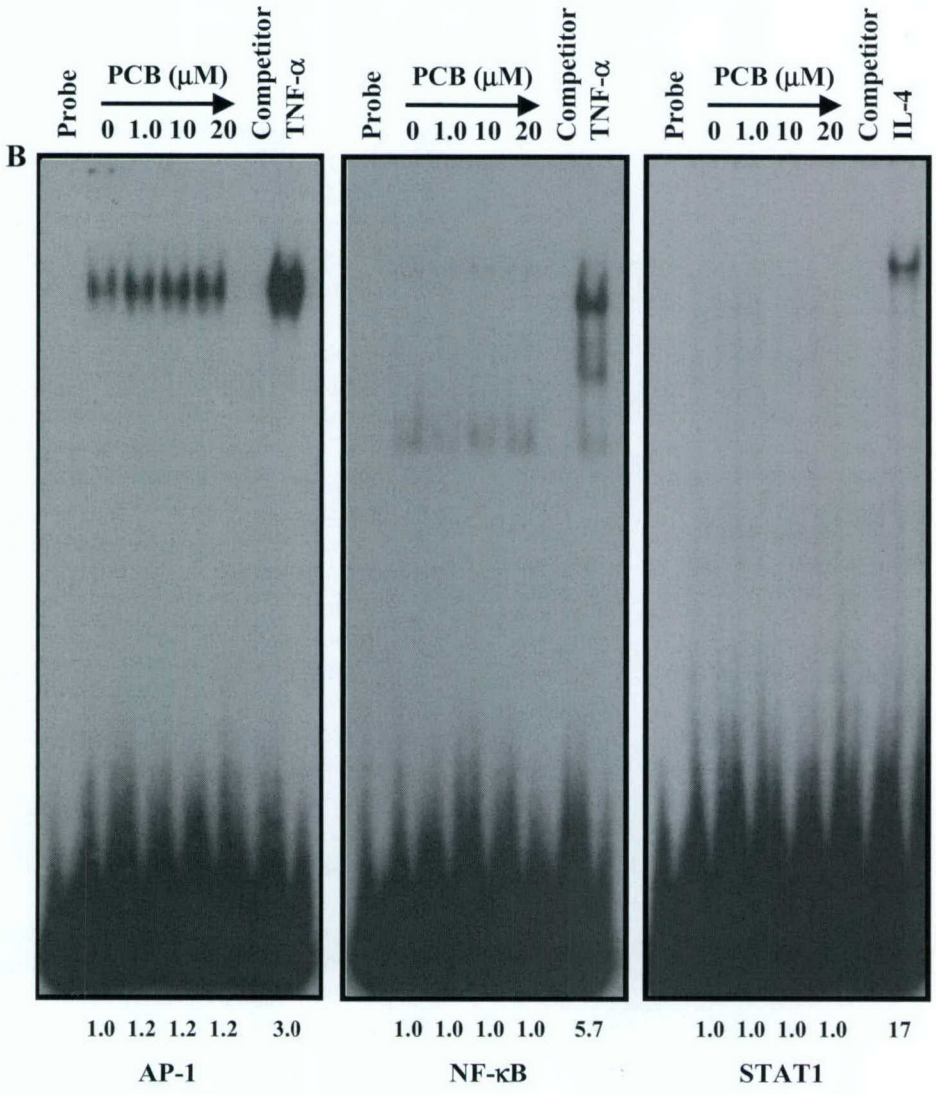

Fig. 4 (*continued*)

at a particular residue, serine 133 (Ser 133), which is required for CREB-mediated transcription. Although the detailed mechanisms by which phosphorylation activates CREB remain unclear, it has been hypothesized that CREB phosphorylation may (1) induce a conformational change that transforms this transcription factor from an inactive to an active form (Gonzalez et al., 1991) and (2) enhance transcription through affecting the ability of CREB to dimerize with different coactivators, such as CREB-binding protein (Gonzalez et al., 1991; Kwok et al., 1994). The best characterized members of the CREB gene family include activating transcription factor (ATF)-1, ATF-2, ATF-3, ATF-4, CREB-1, and CREB-2, and each of the ATF/CREB proteins can bind to CRE in either homodimeric or heterodimeric forms (Shaywitz and Greenberg, 1999).

Previous studies on molecular regulation of apoptosis have demonstrated that the CREB transcription factors may have both antiapoptotic and proapoptotic effects. The antiapoptotic activities of CREB have been observed in a number of different cell and tissue types through a mechanism involving upregulation of antiapoptotic genes such as Bcl-2 and protein kinase B/Akt (Jean et al., 1998; Reusch and Klemm, 2002; Riccio et al., 1999; Somers et al., 1999), indicating that CREB and its associated proteins may function as a survival factor in diverse types of cells. On the other hand, there is a growing line of evidence for proapoptotic effects of CREB. For example, it was demonstrated that CREB phosphorylation mediated by MAPKs can play a critical role in interleukin-1β-induced apoptosis of rat insulin-producing RINm5F cells (Saldeen et al., 2001; Welsh, 1996). Similar effects were observed in the present study in HMEC-1 treated with PCB 104. Thus, it appears that CREB activation and subsequent inhibition or promotion of apoptosis may depend on the type of the apoptotic-inducing stimuli and the specific cell type.

Although the results of the present study convincingly demonstrate that PCB 104 treatment can both activate CREB and induce apoptosis of HMEC-1 (Figs. 2 and 4A),

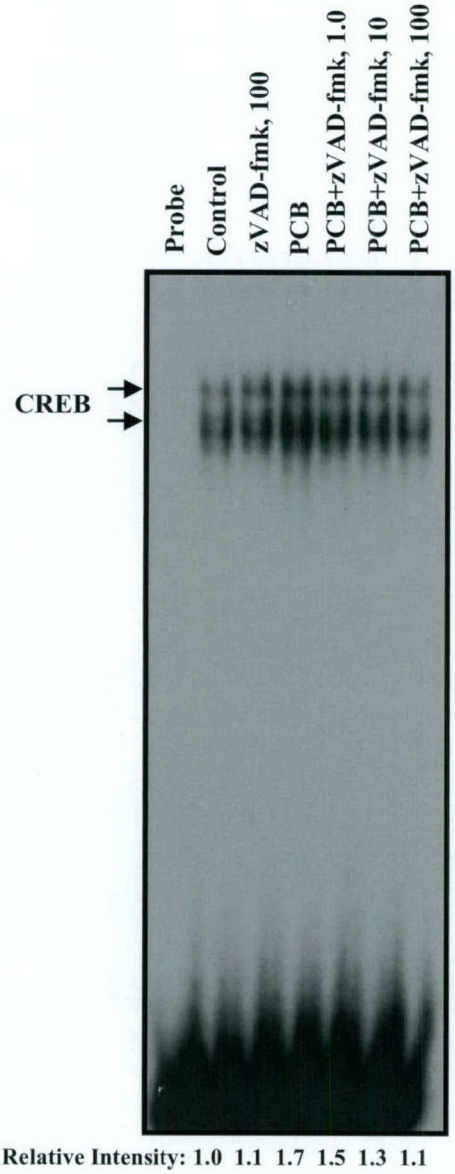

Fig. 5. Caspase inhibitor zVAD-fmk attenuates the CREB DNA-binding activity in PCB 104-stimulated HMEC-1. Cells were pretreated with the indicated amounts of zVAD-fmk (1.0, 10, and 100 μM) for 30 min and then exposed to PCB 104 (20 μM) for 1 h. Following treatment exposures, nuclear extracts were prepared and analyzed by EMSA as described in the legend to Fig. 4A.

the mechanisms of this interrelationship are not fully understood. However, recent data arising from in vitro and in vivo studies demonstrated that CREB can be specifically cleaved by caspases and that CREB cleavage correlates with caspase activity and apoptosis in neuronal cells (Francois et al., 2000). Moreover, it was demonstrated that mildly oxidized low-density lipoprotein can induce apoptosis of human coronary endothelial and smooth muscle cells, the effect that was associated with activation of caspases as well as MAP- and Jun kinases-dependent transcription factors such as p53, ATF-2, ELK-1, AP-1, and CREB (Napoli et al., 2000).

The downstream targets of CREB that may participate in cell death or apoptosis are not fully understood. Nevertheless, evidence indicates that CREB phosphorylation may promote apoptosis via overexpression of proapoptotic genes. For example, CREB has been shown to function as a positive regulator of cyclooxygenase-2 (COX-2) gene expression through a direct binding to the CRE element in the 5′-flanking region of the COX-2 gene (Caivano and Cohen, 2000). Upregulation of COX-2 and the elevation of the COX-2 reaction product, prostaglandin E_2, have been reported to induce caspase-dependent apoptosis in neuronal cells (Takadera et al., 2002). In addition, it also was demonstrated that the induction of COX-2 expression can trigger apoptosis in rat brain in response to kainic acid-induced seizures, a model of hippocampal neurodegeneration (Tocco et al., 1997). These studies support the hypothesis that CREB activation by PCB 104 treatment also may induce proapoptotic gene expression in human microvascular endothelial cells.

In summary, the present study demonstrates that PCB 104 induces apoptosis of human microvascular endothelial cells via the caspase-dependent pathway. In addition, selective activation of transcription factor CREB may be critically involved in the molecular signaling mechanisms of this process.

Acknowledgments

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PCB 104-Induced Proinflammatory Reactions in Human Vascular Endothelial Cells: Relationship to Cancer Metastasis and Atherogenesis

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Polychlorinated biphenyls (PCBs) are widespread environmental contaminants that are known to induce carcinogenic and possibly atherogenic events. Recent evidence suggests that selected PCBs may be potent developmental agents of vascular inflammatory responses by inducing cellular oxidative stress and activating redox-responsive transcription factors. Therefore, the aim of this paper is to investigate PCB-induced proinflammatory reactions in human vascular endothelial cells. To determine the proinflammatory effects, cellular oxidative stress and expression of genes encoding for monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules, such as E-selectin and intercellular adhesion molecule-1 (ICAM-1), were assessed in human umbilical vein endothelial cells (HUVEC) exposed to 2,2′,4,6,6′-pentachlorobiphenyl (PCB 104), a representative of *ortho*-substituted, noncoplanar PCB congeners. PCB 104 increased the oxidative stress in endothelial cells, as determined by the increased 2′,7′-dichlorofluorescein (DCF) and rhodamine 123 fluorescence. In addition, PCB 104 markedly upregulated the expression of MCP-1, E-selectin, and ICAM-1 at both the mRNA and protein levels. These effects were time- and concentration-dependent. The maximum expression of inflammatory genes was observed in endothelial cells exposed to 20 μM of PCB 104 for 1 or 2 h, depending on the specific gene. In addition, PCB 104 elevated the adhesion of THP-1 cells (a human acute monocytic leukemia cell line) to endothelial cell monolayers. These results indicate that PCB 104 is a potent stimulant of inflammatory mediators in human vascular endothelial cells. We hypothesize that these proinflammatory processes may contribute to the development of cancer metastasis and/or atherogenesis in patients exposed to PCBs.

Key Words: **PCB; endothelial cells; inflammation; metastasis; atherosclerosis; vascular disease.**

The formation of blood-borne metastasis is a complex process by which tumor cells spread out from the primary tumor. Evidence indicates that the endothelium is actively involved in the formation of blood-borne metastasis of malignant tumors (Maemura and Dickson, 1994). The process is initiated when tumor cells leave the primary site and invade the vessels to reach the blood stream. The tumor cells can then travel to distant sites via the circulatory system, adhere to the vascular endothelium, penetrate the vessel wall, and establish metastases (Saiki, 1997).

A growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical step in the formation of blood-borne metastasis. It requires the binding of tumor cells to specific adhesion molecules on the surface of endothelial cells. Chemokines, such as monocyte chemoattractant protein-1 (MCP-1) (Youngs *et al.*, 1997) and several adhesion molecules, including E-selectin (Krause and Turner, 1999) and intercellular adhesion molecule-1 (ICAM-1) (Johnson, 1999), may mediate this process. It was shown that the upregulation of endothelial cell adhesion molecules increased the adhesion of tumor cells to the endothelium. This process may initiate the migration of tumor cells through the endothelium into underlying tissues and protect them against destruction by cells of the immune system (Maemura and Dickson, 1994). Expression of chemokines and adhesion molecules is regulated by alterations of the cellular oxidative status through the activation of redox-responsive transcription factors, such as nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) (Collins and Cybulsky, 2001). Thus, increased oxidative stress can modulate the gene expression profile in the vascular endothelium, favoring the induction of inflammatory mediators and thus the stimulation of metastatic processes.

The vascular endothelium forms an interface between the blood and the underlying layers of the vessel walls, and thus it is exposed to a variety of pathophysiological stimuli, such as environmental toxicants, including polychlorinated biphenyls (PCBs). Indeed, endothelial cells may be targets for the bioactivation and toxicity of these compounds (Annas *et al.*, 1998). However, only sparse information is available about the effects of PCBs on endothelial cell metabolism. We show that

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the exposure of endothelial cells to selected PCBs, and especially to PCB 77, can result in the induction of cellular oxidative stress, decrease in cellular antioxidants, and activation of NF-κB (Hennig *et al.*, 2002; Toborek *et al.*, 1995). In addition, selected PCBs elevated the permeability across endothelial monolayers (Toborek *et al.*, 1995). Based on the consensus that cancer metastasis can exploit the mechanisms of endothelial cell activation and the inflammatory responses, we hypothesize that PCB-induced endothelial cell toxicity can result in the development of metastatic processes.

The vascular endothelium plays a critical role not only in cancer metastasis but also in atherogenesis. Endothelial cell dysfunction and the upregulation of inflammatory mediators is one of the main early events in atherogenesis. In fact, recent evidence indicates that atherosclerosis is a chronic inflammatory disease. Our data on PCB-induced toxicity in endothelial cells suggest a possible involvement of this group of compounds in the development of atherosclerosis (Hennig *et al.*, 2002; Toborek *et al.*, 1995). Indeed, several epidemiological studies indicated a strong link between PCB exposure and increased development of heart disease and/or elevated mortality due to atherosclerosis (Gustavsson and Hogstedt, 1997; Hay and Tarrel, 1997). These research findings are supported further by the reports on PCB-induced vascular changes in other species. For example, exposure to PCBs produced vascular lesions in the placental labyrinthine zones of viable fetuses in minks, induced the degeneration of endothelial cells, and induced the formation of thrombi and hemorrhages. The presence of extracellular fluid between the interstitial layer of maternal vessels and the syncytiotrophoblast also indicated compromised endothelial integrity (Backlin *et al.*, 1998).

Because of the potential involvement of PCBs in the induction of oxidative stress and proinflammatory responses, that is, the processes that play important roles in both cancer metastasis and atherogenesis, the aim of this paper is to determine the proinflammatory reactions of PCB 104 in the vascular endothelium. PCB 104 is a typical example of a nonplanar PCB congener with multiple *ortho*-chlorine-substituents. In this paper we provide strong evidence that PCB 104 can induce oxidative stress and the expression of inflammatory mediators in human vascular endothelial cells.

MATERIALS AND METHODS

Cell cultures and PCB 104 treatment. Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described in Toborek et al. (2002). Briefly, isolated cells were seeded into T-75 culture flasks and grown to confluence in M199 medium (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), 30 μg/ml of endothelial cell growth supplement (ECGS; BD Biosciences, San Jose, CA), 50 U/ml of Heparin, 25 mM of HEPES, 100 U/ml of penicillin-streptomycin (Gibco BRL, Grand Island, NY), and 100 U/ml of antibiotics-antimycotics (Gibco BRL, Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were determined to be endothelial by their cobblestone morphology, the expression of the von Willebrand factor (vWF), and the uptake of fluorescently labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes, Eugene, OR). HUVEC from passage 2 were used in all described experiments.

The human monocytic leukemia cell line THP-1 was used to study cell adherence. THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in suspension in RPMI 1640 medium supplemented with 10% FBS, 25 mM of glucose, 10 mM of HEPES, 1.0 mM of sodium pyruvate, 50 μM of 2-mercaptoethanol, 100 U/ml of penicillin, and 100 U/ml of streptomycin.

Serum concentration of PCBs can reach approximately 3 μM in people exposed to these toxicants (Jensen, 1989; Wassermann *et al.*, 1979); however, local levels of PCBs in extracellular space are not known. Therefore, in the present study, the HUVEC were treated with a range of PCB 104 concentrations, such as 1, 10, or 20 μM (AccuStandard, New Haven, CT). A similar experimental design was used in our previously published study (Lee *et al.*, 2003). A stock solution of PCB 104 was prepared in DMSO, and the same amounts of dimethylsulfoxide (DMSO) as in PCB-treated cells were added to control cultures. The basic composition of the experimental medium was the same as that of growth medium, except for the serum concentration, which was lowered to 10%. In selected experiments, tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN) at the concentration of 20 ng/ml was used as a positive control.

Measurement of reactive oxygen species (ROS). The generation of ROS was measured using 2′,7′-dichlorofluorescein (DCF) and rhodamine 123 fluorescence methods. To determine DCF fluorescence, the cells were loaded with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), which freely enters the cells. In the cytoplasm, the ester groups were hydrolyzed by cellular esterases to form dichlorodihydrofluorescein (H_2DCF). H_2DCF was then oxidized by intracellular ROS to highly fluorescent DCF. To measure rhodamine 123 fluorescence, the cells were loaded with dihydrorhodamine 123, which is oxidized by ROS to rhodamine 123 (Negre-Salvayre *et al.*, 2002).

Fluorescence of DCF and rhodamine 123 was assessed as described in Toborek *et al.* (2002) with modifications. Briefly, confluent HUVEC cultures, grown on 24-well cell culture plates, were rinsed three times with Hank's balanced salt solution (HBSS) and incubated with 20 μM of H_2DCF-DA or 5 μM of dihydrorhodamine 123 in HBSS for 30 min at 37°C. Then the cells were rinsed twice with HBSS and incubated with PCB 104 for 30 min in a cell culture incubator. At the end of incubation, the cultures were rinsed twice with HBSS, and 0.5 ml of HBSS was added into each well. The relative fluorescence intensity of the cells was assessed using a fluorescence plate reader. The excitation and emission wavelengths to determine DCF fluorescence were 485 and 530 nm, respectively; and for 123 rhodamine fluorescence they were 488 and 510 nm, respectively.

Reverse transcription and polymerase chain reaction (RT-PCR). RT-PCR was performed as described in Toborek *et al.* (2002). Briefly, total RNA was extracted by TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's guidelines. Reverse transcription was performed at 42°C for 60 min and followed by incubation at 95°C for 5 min. The reaction mixture (20 μl of total volume) consisted of 1 μg of isolated total RNA, 5 mM of $MgCl_2$, 10 mM of Tris–HCl, pH 9.0, 50 mM of KCl, 0.1% Triton X-100, 1 mM of dNTP, 1 unit/μl of recombinant RNasin ribonuclease inhibitor, 15 U/μg of avian myeloblastosis virus (AMV) reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer. For the determination of target genes, specific amplification profiles were used (Table 1). The PCR mixture consisted of a Taq PCR Master Mix (Qiagen, Valencia, CA), 2 μl of the reverse transcription product, and 20 pmol of primer pairs in a total volume of 50 μl. The PCR products were separated by 2% agarose (Invitrogen, Carlsbad, CA) gel electrophoresis, stained with SYBR Gold (Molecular Probes, Eugene, OR) solution for 1 h, and visualized by phosphoimage analysis (FLA-2000, Fuji, Stamford, CN).

Densitometry of each RT-PCR product was performed with *ImageGauge*™ 3.1 software (Fuji, Stamford, CT). Densitometric values of β-actin bands were used to standardize the results. The levels of mRNA were expressed as the ratio of the corresponding gene to β-actin expression.

Enzyme-linked immunosorbent assay (ELISA). MCP-1 concentrations in the cell culture supernatants were determined using the Quantikine® Human

TABLE 1
Sequences of the Primer Pairs Employed in the RT-PCR Reactions[a]

Studied gene	Sequences of the primer pairs (5′–3′) and thermocycling conditions
MCP-1[b]	Forward: CAG CCA GAT GCA ATC AAT GC Reverse: GTG GTC CAT GGA ATC CTG AA Thermocycling: 94°C for 4 min; followed by 94°C for 45 s, 55°C for 45 s, 72°C for 45 s (repeated 22 times); followed by an extension at 72°C for 10 min
ICAM-1 (Staunton *et al.*, 1988)	Forward: GGT GAC GCT GAA TGG GGT TCC Reverse: GTC CTC ATG GTG GGG CTA TGT CTC Thermocycling: 94°C for 5 min; followed by 94°C for 45 s, 60°C for 45 s, 72°C for 60 s (repeated 22 times); followed by an extension at 72°C for 7 min
E-selectin (Meagher *et al.*, 1994)	Forward: CTC TGA CAG AAG AAG CCA AG Reverse: ACT TGA GTC CAC TGA AGC CA Thermocycling: 94°C for 2 min; followed by 94°C for 60 s, 55°C for 60 s, 72°C for 60 s (repeated 26 times); followed by an extension at 72°C for 7 min
β-Actin (Ballester *et al.*, 1998)	Forward: AGC ACA ATG AAG ATC AAG AT Reverse: TGT AAC GCA ACT AAG TCA TA Thermocycling: The same profile and cycle number as the target gene

[a]Preliminary studies showed that amplification was linear within the range of 15–30 cycles for individual genes.
[b]Primer pair purchased from R&D Systems (Minneapolis, MN).

MCP-1 Immunoassay kit (R&D Systems, Minneapolis, MN). Briefly, the HUVEC were cultured to confluence in 6-well plates and treated for 18 h with different doses of PCB 104. At the end of the treatment period, 100-μl aliquots of cell culture media were transferred to anti–MCP-1 antibody-coated wells and incubated for 2 h. Then peroxidase-conjugated secondary polyclonal antibody was added into each well. Following a wash to remove any unbound antibody-enzyme reagents, a substrate solution was added into each well and a reaction was allowed to develop for 20 min. Next, color development was stopped and absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Immunofluorescence analysis (IFA). The HUVEC were grown to confluence on two-chamber culture slides (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) and incubated with 20 μM of PCB 104 or 20 ng/ml of TNF-α for 18 h. The cells were then washed with PBS and fixed with 2% paraformaldehyde in PBS for 20 min. After three consecutive washes to remove paraformaldehyde, the cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS. Unspecific binding was blocked by incubation with 0.1% bovine serum albumin in PBS for 30 min. The cells were then stained for 1 h at room temperature with an anti-human E-selectin (CD62E, 5 μg/ml) or anti-human ICAM-1 (CD54, 1 μg/ml) mouse monoclonal antibody (R&D Systems, Minneapolis, MN), followed by a 1-h incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). The slides were then washed three times with PBS, mounted using a Gel/Mount™ aqueous mounting medium (Biomeda, Foster City, CA), and evaluated using a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments Inc., Melville, NY) at a magnification of ×400.

Cell adhesion assay. The adhesion of THP-1 cells to the HUVEC was assessed according to the method of Braut-Boucher *et al.* (1995) with modifications. Briefly, the HUVEC were grown to confluence on gelatin-coated 24-well plates. The HUVEC were treated with different doses of PCB 104 for 7 h at 37°C and, prior to the adhesion assay, washed three times with HBSS containing 1% BSA (HBSS/BSA).

THP-1 (a human monocytic leukemia cell line) cells were activated with lipopolysaccharide (2 μg/ml, 10-min incubation), washed three times with HBSS/BSA, and suspended in the amount of 1.0×10^6 cells/ml HBSS/BSA. The activated THP-1 cells were labeled with 5 μg/ml of calcein-AM (Calbiochem, La Jolla, CA) by 30-min incubation at 37°C, followed by three washings with HBSS/BSA. Activated and labeled THP-1 cells were then incubated with PCB 104–treated HUVEC for 30 min at 37°C. Nonadherent cells were removed by careful three-time washings with HBSS. The adherence of calcein-labeled THP-1 cells was quantified by fluorescence measurements of endothelial monolayers using an excitation of 490 nm and an emission of 517 nm.

Statistical analysis. Routine statistical analysis of data was completed using *Sigma Stat* 2.0 (SPSS Inc., Chicago, IL). A one-way ANOVA was used to compare the responses among the treatments. The treatment means were compared using Bonferroni's least significant procedure. A statistical probability of $P < 0.05$ was considered significant.

RESULTS

PCB 104 Stimulates Oxidative Stress in Endothelial Cells

Cellular oxidative stress was measured by two fluorescent methods, namely, 2′,7′-DCF and rhodamine 123 fluorescence. These probes are widely used to detect the generation of ROS in viable cells. The effects of 30-min exposure to PCB 104 on DCF and rhodamine fluorescence in endothelial cells are shown in Figure 1, upper and lower panels, respectively. As indicated, PCB 104 at the concentration of 1 μM did not affect cellular oxidative stress. However, PCB 104 at concentrations of 10 μM and higher significantly increased the production of ROS in endothelial cells as measured both by DCF and rhodamine 123 fluorescence. However, the concentration-dependent effects were better reflected by rhodamine 123 fluorescence. Indeed, endothelial cell treatment with 20 μM of PCB 104 significantly increased rhodamine 123 fluorescence as compared to values determined in cultures exposed to 10 μM of this PCB.

PCB 104 Induces MCP-1 Expression in Endothelial Cells

To investigate the effects of PCB 104 on MCP-1 expression, the HUVEC were treated with increasing doses of PCB 104 for

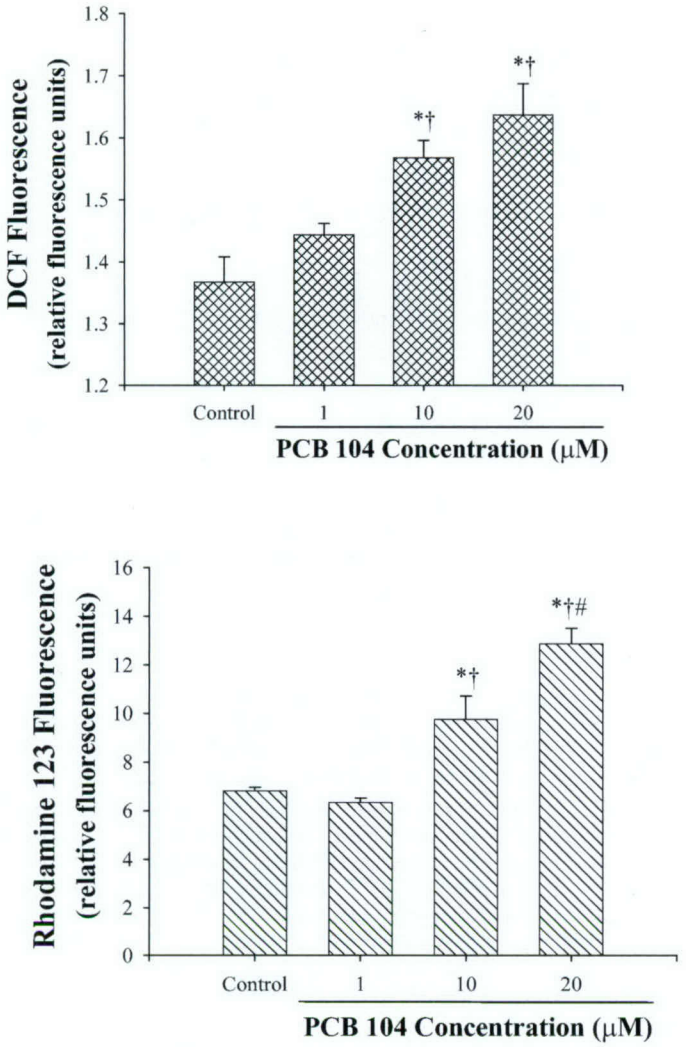

FIG. 1. PCB 104 stimulates DCF fluorescence (upper panel) and 123 rhodamine fluorescence (lower panel) in human endothelial cells. The ells were incubated with the indicated amounts of PCB 104 for 30 min. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104. #Statistically significant as compared to 10-μM PCB 104.

up to 8 h, and the MCP-1 mRNA levels were determined by RT-PCR. The results of these experiments are shown in Figure 2. Figure 2A indicates the concentration-dependent effects of PCB 104 on MCP-1 gene expression. As compared to the controls, PCB 104 at the concentration of 1 μM slightly upregulated MCP-1 mRNA levels. In addition, this expression was further stimulated by higher concentrations of PCB 104. In cells treated with 10 and 20 μM of PCB 104, MCP-1 mRNA levels increased 3.2- and 3.8-fold, respectively. However, the differences in MCP-1 mRNA expression between the cultures treated with 10 and 20 μM of PCB 104 were not significantly different.

Time-dependent effects of 20-μM PCB 104 on MCP-1 mRNA levels are illustrated on Figure 2B. The maximum upregulation of MCP-1 gene expression was observed at 1 h of PCB 104 exposure and then markedly decreased in cultures treated for longer periods of time. However, statistically significant elevations in MCP-1 mRNA levels also were observed after 2 and 8 h of PCB 104 treatments when compared to the control levels.

To establish whether PCB 104 induced an increase in MCP-1 mRNA levels that can be translated into elevated protein expression, a sandwich ELISA was employed to determine MCP-1 protein production in cultures treated with increasing doses of PCB 104 for 18 h. Because MCP-1 is secreted from the cells, the determinations were performed in cell culture media. The results of these experiments are shown in Figure 2C. Compared to the control or 1-μM PCB 104, exposure to 10- and 20-μM PCB 104 significantly increased the MCP-1 protein levels.

PCB 104 Upregulates E-Selectin and ICAM-1 Expression in Endothelial Cells

Among adhesion molecules, E-selectin and ICAM-1 play an important role in mediating the adhesion of both tumor cells and leukocytes to the vascular endothelium. Therefore, the effects of PCB 104 on the expression of these adhesion molecules were assessed in the present study. The effects of PCB 104 on E-selectin and ICAM-1 expression are shown in Figures 3 and 4, respectively. PCB 104 at concentration of 1 μM did not affect the expression of adhesion molecules. However, a statistically significant increase in both E-selectin (Fig. 3A) and ICAM-1 (Fig. 4A) mRNA levels was observed in cells treated with PCB 104 at concentrations of 10 or 20 μM when compared to controls as well as cultures exposed to 1 μM PCB 104. In addition, the level of E-selectin mRNA expression was significantly higher in cells treated with 20 μM PCB 104 as compared to that in cultures incubated with 10-μM PCB 104.

Time-dependent studies revealed that a 2-h exposure to 20 μM PCB 104 induced the maximum effect on E-selectin mRNA expression, whereas there were no significant changes at 1-h exposure time. Markedly increased levels of E-selectin mRNA also were observed in endothelial cells treated with PCB 104 for 4 h. However, after a longer exposure time, such as 8 h, E-selectin mRNA expression returned to the control values (Fig. 3B). ICAM-1 mRNA levels already were elevated as the result of a 1-h treatment with 20-μM PCB 104 and remained at the same levels in cells treated for 2 h (Fig. 4B). Longer exposure time gradually decreased ICAM-1 mRNA expression to near control values at 8 h of PCB 104 treatment.

To determine whether increased levels of E-selectin and ICAM-1 mRNA were associated with elevated protein levels, expression of these adhesion molecules on the surface of endothelial cells was determined by immunocytochemistry. Consistent with gene expression studies, treatment with 20-μM PCB 104 for 18 h markedly upregulated the protein expression of both E-selectin and ICAM-1 (middle panels on Figs. 3C and

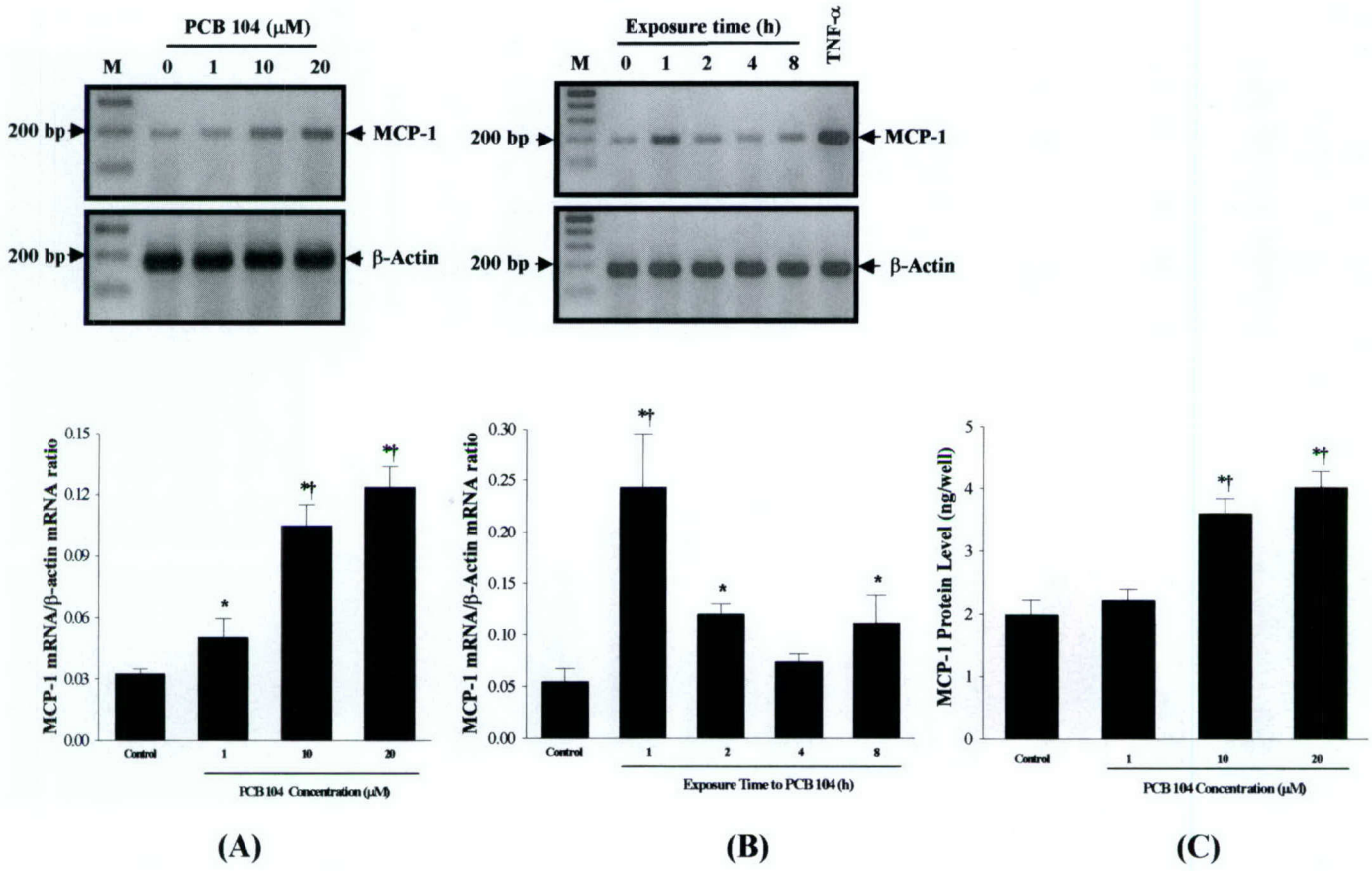

FIG. 2. (A) Concentration-dependent effects of PCB 104 on the induction of MCP-1 mRNA levels in human endothelial cells. The cells were incubated with increasing concentrations of PCB 104 for 2 h. Following treatment exposures, RNA was isolated, and the expression of MCP-1 and β-actin gene was analyzed by RT-PCR (upper panel), followed by densitometric measurements. The experiments were repeated four times, and the ratios of MCP-1 to β-actin mRNA levels were statistically analyzed (lower panel). The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104. (B) Time-dependent effects of PCB 104 on the induction of MCP-1 mRNA levels in human endothelial cells. The cells were incubated with 20-μM PCB 104 for up to 8 h. The expression of MCP-1 mRNA was determined as in the caption of Figure 2A. *Upper* panel: Representative RT-PCR analysis of PCB 104-induced MCP-1 expression. Lower panel: Statistical analysis of densitometric analysis of the band corresponding to the MCP-1 mRNA level. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to other PCB 104 exposure times. M, molecular weight markers (100-bp DNA ladder). (C) Concentration-dependent effects of PCB 104 on the induction of MCP-1 protein levels in human endothelial cells. The cells were incubated with increasing concentrations of PCB 104 for 18 h. Following treatment exposures, MCP-1 protein was measured by ELISA in cell culture media. The data shown are the means ± SD of six determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104.

4C, respectively). Treatments with TNF-α at a concentration of 20 ng/ml were used as positive controls in these experiments (right panels on Figs. 3C and 4C).

Exposure to PCB 104 Upregulates Cell Adhesion to Endothelial Cell Monolayers

The adherence of THP-1 cells (a human acute monocytic leukemia cell line) to PCB 104–treated HUVEC was assessed to determine whether the induction of inflammatory mediators observed in PCB 104–treated endothelial cells can stimulate cell adhesion. Because of their cancer and leukocyte linkage, THP-1 cells are ideally suited for the experiments related to cancer metastasis and atherogenesis. Consistent with the data on MCP-1 and adhesion molecules, PCB 104 markedly and in a concentration-dependent manner stimulated the adherence of THP-1 cells to endothelial monolayers (Fig. 5). The maximum effect on cell adhesion was observed in the HUVEC treated with 20-μM PCB 104.

DISCUSSION

Different PCB structures interact specifically with different cellular targets. In our earlier studies, we indicated that coplanar PCBs that are aromatic hydrocarbon receptor (AhR) ligands, such as PCB 77, PCB 126, and PCB 169, can activate endothelial cells both *in vitro* and *in vivo* (Hennig *et al.*, 2002).

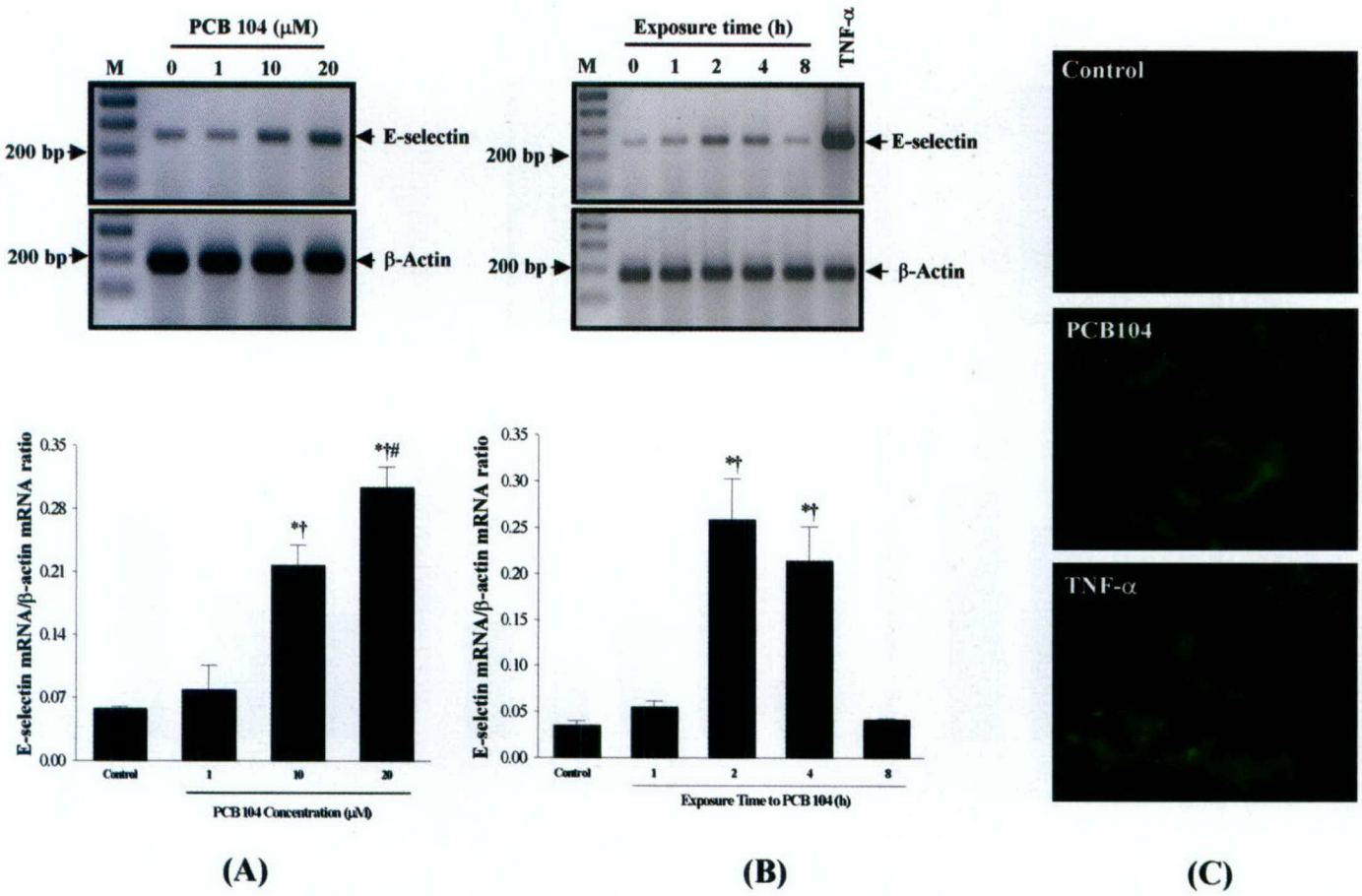

FIG. 3. (A) Concentration-dependent effects of PCB 104 on the induction of E-selectin mRNA levels in human endothelial cells. The experiments were performed as described in the caption of Figure 2A. Upper panel: Rrepresentative RT-PCR analysis, as visualized by phosphoimaging technology. Lower panel: Statistical analysis of densitometric analysis of the band corresponding to the E-selectin mRNA level. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104. #Statistically significant as compared to 10-μM PCB 104. (B) Time-dependent effects of PCB 104 on the induction of E-selectin mRNA levels in human endothelial cells. The experiments were performed as described in the caption of Figure 2B. Upper panel: Representative RT-PCR analysis, as visualized by phosphoimaging technology. Lower panel: Statistical analysis of densitometric analysis of the band corresponding to E-selectin mRNA level. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to other PCB 104–exposure times. (C) Effect of PCB 104 on the induction of E-selectin protein in human endothelial cells. The cells were incubated with 20-μM PCB 104 for 18 h, and E-selectin protein expression was determined on the surface of the endothelial cells by immunocytochemistry. Left panel: Control cells. Middle panel: Cells treated with 20-μM PCB 104. Right panel: Cells treated with TNF-α at the concentration of 20 ng/ml (positive control).

The role of the AhR activation in PCB-induced cytotoxicity was confirmed in several other research reports. For example, it was shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can activate NF-κB and AP-1 through the CYP1A1-dependent and AhR complex–dependent oxidative signals (Puga *et al.*, 2000). In addition, it was demonstrated that PCB-induced oxidative stress can be related to the uncoupling of CYP1A1 reactions (Schlezinger *et al.*, 1999).

PCB 104 is an example of a group of highly *ortho*-chlorine-substituted, non-coplanar PCB congeners. Such PCBs are not typical AhR or constitutive androstane receptor (CAR) agonists. The biological effects of these PCBs include neurotoxicity, estrogenicity, and insulin release, as well as altered regulation of intracellular calcium and signal transduction mechanisms (Fischer *et al.*, 1998; Kodavanti and Tilson, 2000). In addition, it was demonstrated that PCB 104 can induce cellular apoptosis through the caspase-mediated mechanism (Lee *et al.*, 2003; Shin *et al.*, 2000). In this paper we provide consistent evidence that PCB 104 also can markedly stimulate the production of proinflammatory responses in endothelial cells.

It is generally accepted that the induction of inflammatory responses is mediated by alterations of the redox status of the cells. Therefore, to address this possibility, the oxidative status of endothelial cells treated with PCB 104 was measured by DCF and rhodamine 123 fluorescence. These probes can detect a broad spectrum of ROS and oxidizing reactions. However, it should be pointed out that neither of these probes detects

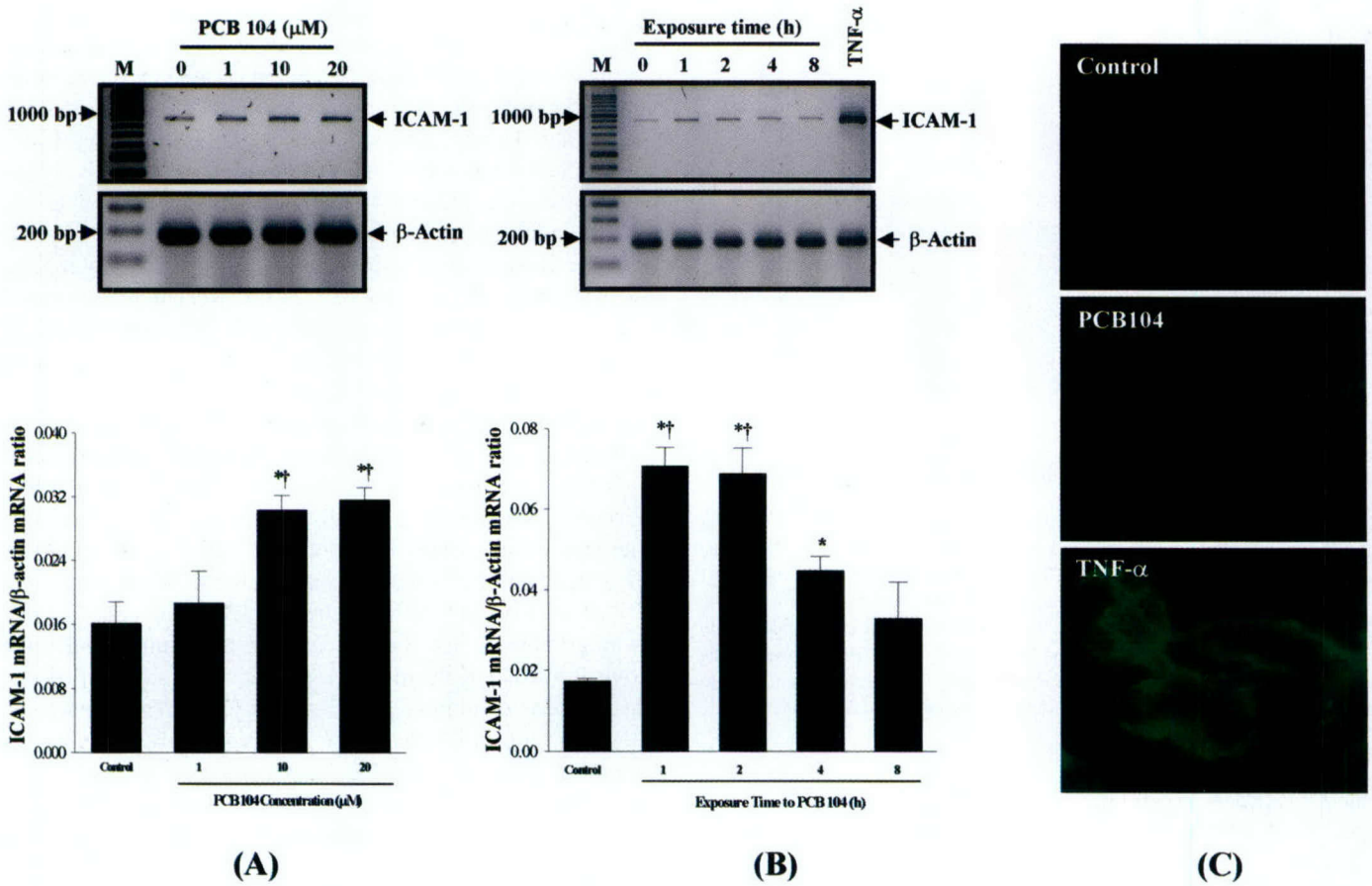

FIG. 4. (A) Concentration-dependent effects of PCB 104 on the induction of ICAM-1 mRNA levels in human endothelial cells. The experiments were performed as described in the caption of Figure 2A. Upper panel: Representative RT-PCR analysis, as visualized by phosphoimaging technology. Lower panel: Statistical analysis of densitometric analysis of the band corresponding to the ICAM-1 mRNA level. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104. (B) Time-dependent effects of PCB 104 on the induction of ICAM-1 mRNA levels in human endothelial cells. The experiments were performed as described in the caption of Figure 2B. Upper panel: Representative RT-PCR analysis, as visualized by phosphoimaging technology. Lower panel: Statistical analysis of densitometric analysis of the band corresponding to the ICAM-1 mRNA level. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to other PCB 104 exposure times. (C) Effect of PCB 104 on the induction of ICAM-1 protein in human endothelial cells. The cells were incubated with 20-μM PCB 104 for 18 h, and ICAM-1 protein expression was determined on the surface of endothelial cells by immunocytochemistry. Left panel: Control cells. Middle panel: Cells treated with 20-μM PCB 104. Right panel: Cells treated with TNF-α at the concentration of 20 ng/ml (positive control).

superoxide anion radicals. DCF fluorescence is localized in the cytosol; in contrast, rhodamine 123 fluorescence is sequestered in the mitochondria (Negre-Salvayre *et al.*, 2002). We indicated that treatment of endothelial cells with increasing doses of PCB 104 markedly elevated the oxidative stress in cultured endothelial cells.

The results of the present study further demonstrated that exposure of human endothelial cells to PCB 104 can markedly induce the expression of inflammatory mediators, such as MCP-1, E-selectin, and ICAM-1, both at mRNA and protein levels. The effects on mRNA levels were very early events, with the maximum increase observed already after a 1- or 2-h treatment. Although this early elevation gradually decreased in cultures exposed to PCB 104 for a longer period of time, it was sufficient to induce protein levels of inflammatory mediators that were measured after an 18-h exposure.

MCP-1 is a member of the CC chemokine family, and it stimulates the chemotaxis and transmigration of monocytes, lymphocytes, and granulocytes (Mukaida *et al.*, 1998). An increased production of MCP-1 is associated with a variety of processes, including cancer metastasis (Amann *et al.*, 1998; Hefler *et al.*, 1999; Youngs *et al.*, 1997) and early stages of atherosclerosis (Boring *et al.*, 1998). At least two distinct mechanisms may be involved in the prometastatic effects of MCP-1. First, MCP-1 can exert direct chemotactic effects on tumor cells, as was shown using MCF-7 cells, a cell line obtained from human breast carcinoma (Youngs *et al.*, 1997). This chemotactic influence of MCP-1 on tumor cells is medi-

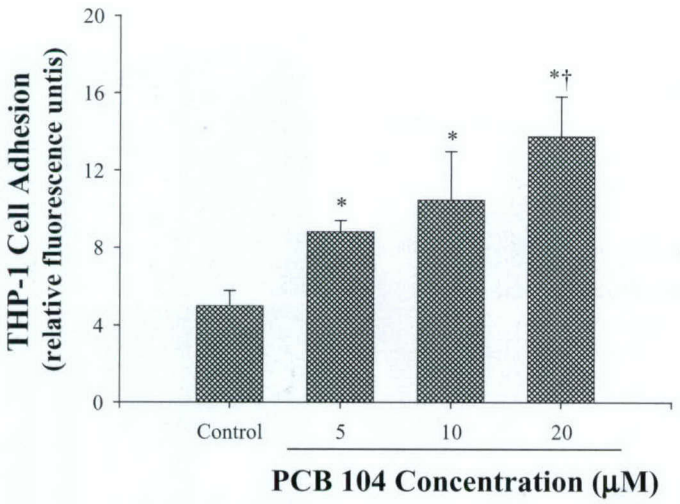

FIG. 5. Concentration-dependent effects of PCB 104 on the adhesion of THP-1 cells (human monocytic leukemia cell line) to endothelial cells. The endothelial cells were treated with 20-μM PCB 104 for 7 h, while the THP-1 cells were activated with lipopolysaccharide (LPS) and labeled with calcein/AM. To perform the adhesion assay, the endothelial cells were incubated with activated and labeled THP-1 cells for 30 min at 37°C. The data shown are the means ± SD of four determinations. *Statistically significant as compared to the control group. †Statistically significant as compared to 1-μM PCB 104.

ated by a receptor-stimulated signaling pathway. Thus, it appears that MCP-1 can directly attract tumor cells and induce tumor cell migration across the vascular endothelium with the subsequent generation of tumor metastasis. A second mechanism by which MCP-1 may stimulate the development of cancer metastasis may be related to its chemotactic effects toward leukocytes (Mukaida *et al.*, 1998). Activated leukocytes can migrate across the endothelium and degrade extracellular matrix proteins, which separate the endothelium from the underlying layers of the vascular wall. Such a process can markedly facilitate the invasion of tumor cells, a process associated with the development of metastasis. The role of MCP-1 in tumor metastasis has been supported by the observations that the levels of this chemokine were elevated in the serum of ovarian cancer patients (Hefler *et al.*, 1999) and in the urine of patients with bladder cancer (Amann *et al.*, 1998). In addition, the urinary MCP-1 levels were strongly correlated with tumor stage, grade, and distant metastasis (Amann *et al.*, 1998).

Evidence indicates that MCP-1 also can play an important role in atherogenesis. To support its role in the initiation and development of atherosclerosis, it was shown that MCP-1 deficiency significantly reduced atherosclerosis in low-density lipoprotein (LDL) receptor-deficient mice fed a high cholesterol diet (Gu *et al.*, 1998). In a similar study, the selective absence of CCR2, the receptor for MCP-1, markedly decreased atherosclerotic lesion formation in apolipoprotein (apo) E-deficient mice (Boring *et al.*, 1998).

PCB 104-induced overexpression of adhesion molecules may also play a role in vascular pathologies associated with cancer metastasis and/or atherosclerosis. For example, convincing experimental data have been generated on the involvement of E-selectin in breast and colon cancer metastasis (Krause and Turner, 1999). Several glycoprotein ligands also have been identified on the surface of colon cancer cells, which serve as specific receptors for E-selectin (Tomlinson *et al.*, 2000). In addition, circulating levels of this adhesion molecule were identified as useful clinical markers of tumor progression and metastasis (Alexiou *et al.*, 2001). Recent experimental evidence also indicated that the inhibition of E-selectin–mediated cancer cell adhesion may be an efficient strategy to inhibit cancer metastasis (Khatib *et al.*, 2002).

In addition to its role in cancer metastasis, overexpression of E-selectin is associated with the development of atherosclerosis. During atherogenesis, the migration of leukocytes through the vascular endothelium initially involves relatively transient adherence of leukocytes to endothelial cells, which results in leukocytes "rolling" over the endothelium (McIntyre *et al.*, 1997). This process is followed by firm leukocyte adhesion and transmigration across the vascular endothelium. Leukocyte rolling is mediated by the overexpression of adhesion molecules of the selectin family, such as E-selectin. The importance of the adhesion molecules of the selectin family in the development of atherosclerosis has been confirmed in studies that demonstrated the presence of both E- and P-selectin on the surface of endothelial cells overlying atherosclerotic plaques (Wood *et al.*, 1993).

ICAM-1 is an adhesion molecule of the immunoglobulin superfamily critically involved in both cancer metastasis and atherogenesis. To support the role of ICAM-1 in cancer metastasis, it was demonstrated that serum levels of soluble ICAM-1 (sICAM-1) were elevated in patients with non-small–cell lung cancer and correlated with the tissue expression of ICAM-1 and tumor stage (Grothey *et al.*, 1998). In addition, metastatic lung cancer was associated with higher sICAM-1 as compared to localized tumors (Grothey *et al.*, 1998), and the highest levels of sICAM-1 were observed in patients with liver metastasis (Sprenger *et al.*, 1997). ICAM-1 expression also correlated with progression of malignant melanoma (Hakansson *et al.*, 1999) and renal cell carcinoma (Tanabe *et al.*, 1997). The role of ICAM-1 in tumor cell metastasis was confirmed by the observation that antisense ICAM-1 oligonucleotides decreased the metastasis of malignant melanoma by approximately 50% (Miele *et al.*, 1994).

In the development of atherosclerosis, ICAM-1 stimulates firm adhesion of leukocytes to the vascular endothelium. ICAM-1 is expressed at low levels on the surface of nonstimulated endothelial cells. In addition, stimuli such as TNF-κ, IL-1, interferon-γ (Dustin *et al.*, 1986), or shear stress (Nakashima *et al.*, 1998) can markedly induce the expression of this adhesion molecule. The stimulatory involvement of hemodynamic stress in the upregulation of ICAM-1 may play an important role in the development of atherosclerosis in hyper-

tension. ICAM-1 is markedly expressed in the early stages of atherosclerosis, and it stimulates the adhesion of monocytes and T lymphocytes. The significance of this adhesion molecule in atherosclerosis was confirmed in clinical studies, which determined elevated levels of sICAM-1 in asymptomatic patients who are prone to develop cardiovascular disease (Ridker *et al.*, 1998).

In summary, the present study indicates that PCB 104, that is, a highly *ortho*-chlorine–substituted, non-coplanar PCB congener, can induce profound vascular effects, as demonstrated by the increase in cellular oxidation and upregulation of MCP-1, E-selectin, and ICAM-1. These effects were both time- and concentration-dependent. In addition, the upregulation of these inflammatory mediators was associated with the increased adhesion of THP-1 cells to vascular endothelial cells. Such PCB 104–induced vascular pathology can participate in the development of cancer metastasis and/or atherosclerosis.

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Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells[1–3]

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ABSTRACT

Background: Activation of the vascular endothelium by dietary fatty acids may be among the most critical early events in the development of atherosclerosis. However, the specific effects of fatty acids on inflammatory responses in endothelial cells are not fully understood.

Objective: The present study focused on the induction of inflammatory genes in human endothelial cells exposed to individual dietary fatty acids. Because of the significance of nuclear factor κB (NF-κB) and activator protein 1 (AP-1) in the regulation of inflammatory gene expression, we also determined the effects of fatty acids on NF-κB and AP-1 transcriptional activation.

Design: Human umbilical vein endothelial cells were exposed to dietary mono- and polyunsaturated 18-carbon fatty acids. Transcriptional activation of NF-κB and AP-1 was determined in human umbilical vein endothelial cells transfected with reporter constructs regulated by these transcription factors. Induction of the inflammatory genes was studied by use of reverse transcriptase–polymerase chain reaction.

Results: Of the fatty acids studied, linoleic acid stimulated NF-κB and AP-1 transcriptional activation the most. In addition, treatment with this fatty acid markedly enhanced messenger RNA levels of tumor necrosis factor α, monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1. Treatment with linolenic acid stimulated only a moderate induction of the genes encoding for these inflammatory mediators, and exposure to oleic acid either had no effect or resulted in decreased inflammatory gene messenger RNA. In addition, exposure to both linoleic and linolenic acids strongly stimulated induction of the phospholipid hydroperoxide glutathione peroxidase gene.

Conclusion: Specific unsaturated dietary fatty acids, particularly linoleic acid, can selectively stimulate the development of a proinflammatory environment within the vascular endothelium. *Am J Clin Nutr* 2002;75:119–25.

KEY WORDS Fatty acids, inflammatory genes, transcription factors, human endothelial cells, atherosclerosis, nuclear factor κB, activator protein 1

INTRODUCTION

Activation or dysfunction of the vascular endothelium is one of the first events in the development of atherosclerosis (1, 2), and

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selected dietary fatty acids may be among the most critical factors that induce these processes. For example, lipids, including selective fatty acids, may cause injury to the endothelium (reviewed in reference 3). It has been proposed that hydrolysis of triacylglycerol-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions near the endothelium (4, 5). In support of this notion, it was shown that lipoprotein lipase activity is increased in atherosclerotic lesions (5–7). Lipoprotein lipase–derived remnants of lipoproteins isolated from hypertriglyceridemic subjects as well as selective unsaturated fatty acids can disrupt endothelial integrity (8, 9). Because the lipid composition of plasma and tissues is closely related to dietary fat intake (10), exposure of endothelial cells to individual fatty acids can be directly influenced by the types of fatty acids consumed in the diet (10, 11).

Strong evidence indicates that exposure to selected dietary unsaturated 18-carbon fatty acids can directly affect endothelial cell metabolism. Significant amounts of data have been accumulated to show that linoleic acid (18:2n−6) can induce marked injury to endothelial cells. For example, it was reported that this fatty acid can disrupt endothelial cell integrity, alter functions of gap-junctional proteins (12), increase concentrations of intracellular calcium, and induce cellular oxidative stress (13). In addition, the treatment of endothelial cells with linoleic acid and tumor necrosis factor α (TNF-α) can activate caspase 3 activity and induce apoptotic cell death (14, 15). The role of other dietary unsaturated 18-carbon fatty acids in endothelial cell metabolism is less well understood. However, evidence indicates that dietary oleic acid can protect endothelial cells against hydrogen peroxide–induced oxidative stress (16) and reduce the susceptibility of LDLs to oxidative modifications (17).

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Atherosclerosis is an inflammatory disease of the vascular wall (18). Inflammatory reactions in endothelial cells are regulated primarily through the production of chemokines [eg, monocyte chemoattractant protein 1 (MCP-1)], inflammatory cytokines (eg, TNF-α), and adhesion molecules [eg, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)]. Expression of these inflammatory mediators and their effects are closely interrelated. In addition, overexpression of MCP-1 (19), TNF-α (20), and ICAM-1 and VCAM-1 (21) is a common feature of atherosclerotic processes.

Inflammatory genes, such as those encoding for MCP-1, TNF-α, ICAM-1, and VCAM-1, are regulated by a variety of transcription factors (2, 22). It appears that nuclear factor κB (NF-κB) and activator protein 1 (AP-1) play critical roles in these regulatory processes. The binding sites for these transcription factors were identified in the promoter regions of various inflammatory genes (22–25), and increased amounts of NF-κB were found in atherosclerotic vessels (26, 27). In addition, selected fatty acids, such as linoleic acid, can activate NF-κB in endothelial cells (13). Moreover, effects mediated by NF-κB and AP-1 appear to be interrelated. For example, it was shown that TNF-α–mediated induction of VCAM-1 expression requires both activated NF-κB and AP-1 (23).

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant enzyme involved in detoxification of lipid hydroperoxides in cellular membranes and lipoproteins (28). Thus, this enzyme may play a critical role in antioxidant protection against oxidative stress induced by unsaturated fatty acids.

Although it is known that selected fatty acids can induce oxidative stress and activate transcription factors responsive to oxidative stress (13), the specific effects of unsaturated fatty acids on inflammatory responses in endothelial cells are not fully understood. Therefore, the focus of the present study was to examine the induction of the inflammatory genes in human endothelial cells exposed to specific 18-carbon, mono- and polyunsaturated fatty acids. In addition, because of the significance of NF-κB and AP-1 in the regulation of the inflammatory genes, the effects of unsaturated fatty acids on the activity of these transcription factors were also determined.

MATERIALS AND METHODS

Human umbilical vein endothelial cell cultures and fatty acid treatments

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (29) and cultured in enriched M199 medium, which included 25 mmol HEPES/L, 54.3×10^3 U heparin/L, 2 mmol L-glutamine/L, 1 μmol sodium pyruvate/L, 200×10^3 U penicillin/L, 200 mg streptomycin/L, 0.25 mg amphotericin B/L (GibcoBRL, Grand Island, NY), 0.04 g endothelial cell growth supplement/L (Becton Dickinson, Bedford, MA), and 20% fetal bovine serum (HyClone, Logan, UT). Cells were determined to be endothelial in origin by their cobblestone morphology and uptake of fluorescently labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR). All experiments were performed on cells from passage 2. Confluent cell cultures were treated with ≤180 μmol/L of oleic acid (18:1n−9), linoleic acid, or linolenic acid (18:3n−3) (Nu-Chek Prep, Elysian, MN). Fatty acid–enriched experimental media were prepared as described earlier (9).

To study the dose-dependent effects of specific unsaturated fatty acids on messenger RNA (mRNA) levels of genes critical in the endothelial cell inflammatory response, HUVECs were exposed to 60, 90, and 180 μmol fatty acids/L. Preliminary experiments showed that fatty acids consistently exerted a maximum effect on inflammatory gene induction at the concentration of 90 μmol/L. Therefore, experiments with 180 μmol fatty acids/L were discontinued and data are presented only from studies in which HUVECs were exposed to 60 and 90 μmol unsaturated fatty acids/L.

Transfections and reporter gene assay

Transfections were performed as described earlier (30). Briefly, HUVECs were seeded in 12-well plates and grown to 50–60% confluency in normal growth medium. Then, aliquots of normal M199 medium were mixed with 36 mg/L of a liposome pFx-7 (Invitrogen, Carlsbad, CA) and with 10 mg/L of NF-κB– or AP-1–responsive plasmids (pNFκB-Luc or pAP1-Luc, respectively) containing a luciferase reporter gene (Stratagene, La Jolla, CA). The transfection mixtures were incubated at 37°C for 30 min to allow DNA-lipid complexes to form. Endothelial cell cultures were washed with M199 medium to remove serum, and 1 mL transfection solution was added for 1.5 h to each well of the 12-well plate. After incubation, transfection solutions were aspirated and replaced with growth medium for 24 h. Then, transfected cultures were treated with specific unsaturated fatty acids for 24 h. Control groups consisted of transfected HUVEC cultures that were not exposed to fatty acids.

Luciferase activity was measured by use of the Luciferase Assay System (Promega, Madison, WI). Briefly, culture media were removed and HUVECs were washed with phosphate-buffered saline and incubated with cell culture lysis reagent. Cell lysates were centrifuged ($12000 \times g$, 2 min, 4°C) to remove membrane debris, and 10 μL of the cell extracts was mixed with 100 μL luciferase assay reagent containing luciferin and ATP in a luminometer with automatic injection. Values are expressed in relative light units (RLU)/μg protein.

Transfection efficiency was monitored as described earlier (30) by transfection of endothelial cells with the VR-3301 vector, which contains human placental alkaline phosphatase as the reporter gene. Under the described conditions, transfection efficiency was determined to be 32% (30). All transfection studies were repeated 3 times by using 6 wells in 12-well plates per experimental group.

Reverse transcriptase–polymerase chain reaction analyses

Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described earlier (29, 31). Briefly, treated HUVECs were lysed and the total RNA was extracted with use of RNA STAT-60 (Tel-TEST, Inc, Friendswood, TX) according to the procedure supplied by the manufacturer. Isolated RNA was quantitated by measuring absorbance at 260 nm. A standard reverse transcription reaction was performed at 42°C for 60 min in 20 μL of 5 mmol $MgCl_2$/L; 10 mmol tris-Cl/L, pH 9.0; 50 mmol KCl/L; 0.1% Triton X-100; 1 mmol dNTP/L; 1×10^6 U recombinant RNasin ribonuclease inhibitor/L; 15×10^6 U AMV reverse transcriptase/L; and 0.5 μg oligo$(dT)_{15}$ primer (Promega). The sequences of the primer pairs used for PCR amplification of the studied genes are shown in **Table 1**. For quantitation, levels of mRNA of the studied inflammatory genes

TABLE 1
Sequences of the primer pairs used in the reverse transcriptase–polymerase chain reactions[1]

Studied mediator	Sequence of the primer pairs (5′–3′)
MCP-1[2]	
Forward	CAG CCA GAT GCA ATC AAT GC
Reverse	GTG GTC CAT GGA ATC CTG AA
TNF-α[2]	
Forward	GTG ACA AGC CTG TAG CCC A
Reverse	ACT CGG CAA AGT CGA GAT AG
ICAM-1	
Forward	GGT GAC GCT GAA TGG GGT TCC
Reverse	GTC CTC ATG GTG GGG CTA TGT CTC
VCAM-1[2]	
Forward	ATG ACA TGC TTG AGC CAG G
Reverse	GTG TCT CCT TCT TTG ACA CT
PHGPx	
Forward	TGT GCG CGC TCC ATG CAC GAG T
Reverse	AAA TAG TGG GGC AGG TCC TTC TCT
β-Actin	
Forward	AGC ACA ATG AAG ATC AAG AT
Reverse	TGT AAC GCA ACT AAG TCA TA

[1] ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; PHGPx, phospholipid hydroperoxide glutathione peroxidase; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1.

[2] Primer pairs purchased from R&D Systems, Minneapolis.

and the gene encoding for PHGPx were related to β-actin mRNA. The PCR mixture consisted of 2 μL of a product of the reverse transcription reaction, a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), and 20 pmol of primer pairs in a total volume of 50 μL. For each individual gene, a linear range of PCR amplification was established and the induction of the target gene was studied within the range.

The following thermocycling conditions were used to determine the induction of the genes encoding for the studied inflammatory mediators:

MCP-1: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 25 times); followed by an extension at 72 °C for 10 min;

TNF-α: 94 °C for 4 min; followed by 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s (repeated 28 times); followed by an extension at 72 °C for 10 min;

ICAM-1: 94 °C for 4 min; followed by 94 °C for 45 s, 60 °C for 45 s, 72 °C for 60 s (repeated 28 times); followed by an extension at 72 °C for 7 min;

VCAM-1: 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s (repeated 25 times); and

PHGPx: 94 °C for 4 min; followed by 94 °C for 40 s, 66 °C for 60 s, 72 °C for 2 min (repeated 20 times); followed by an extension at 72 °C for 7 min.

Induction of the β-actin gene was determined by using the same number of cycles and thermocycling conditions as for the target genes. Under these RT-PCR conditions, the β-actin transcript increased linearly in the range of 15–40 PCR cycles.

PCR products were separated by 2%-agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes), and visualized by using phosphorimaging technology (FLA-2000; Fuji, Stamford, CT). The relative intensity of fluorescence (ratio of the intensity of the band corresponding to the target gene to that corresponding to the β-actin gene) was quantified with IMAGE GAUGE 3.0 software (Fuji) and expressed as average pixel intensity. Experiments were repeated 4 times on different days, and the values of relative fluorescence from the 4 experiments were statistically analyzed.

Statistical analysis

Statistical analysis was performed by using SYSTAT 8.0 (SPPS Inc, Chicago). One-way analysis of variance was used to compare mean values among the treatments. When the overall F values were significant, analysis of variance was followed by post hoc Bonferroni tests to compare means from different treatments. A P value <0.05 was considered significant.

RESULTS

Unsaturated fatty acids selectively induce NF-κB and AP-1 transcriptional activation

The effects of specific unsaturated fatty acids on NF-κB transcriptional activation are shown in **Figure 1**A. Treatment of

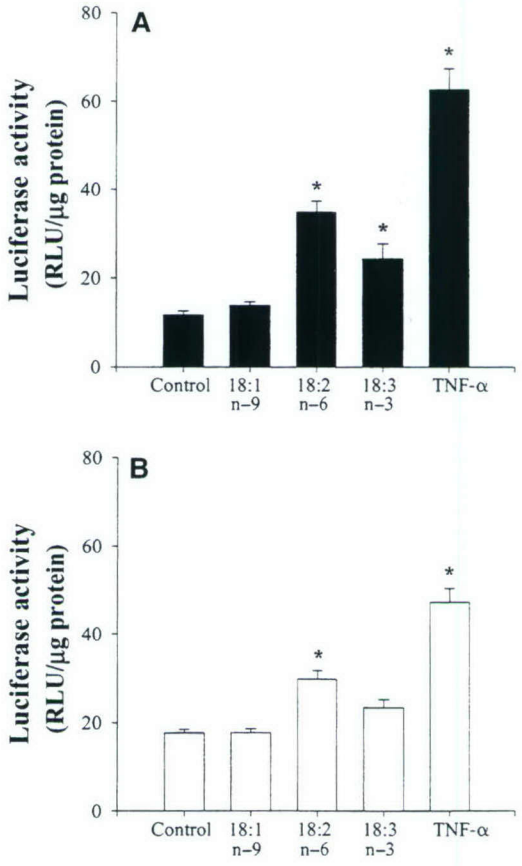

FIGURE 1. Mean (±SEM) fatty acid–induced nuclear factor κB (NF-κB)–related (A) and activator protein 1 (AP-1)–related (B) transcription in human endothelial cells. Transcriptional activation was measured by luciferase activity in human umbilical vein endothelial cells transfected with an NF-κB–responsive or AP-1–responsive luciferase reporter construct and exposed to specific unsaturated fatty acids (90 μmol/L) for 24 h. Tumor necrosis factor α (TNF-α) treatment was used as a positive control. RLU, relative light units. *Significantly different from control cultures, $P < 0.05$.

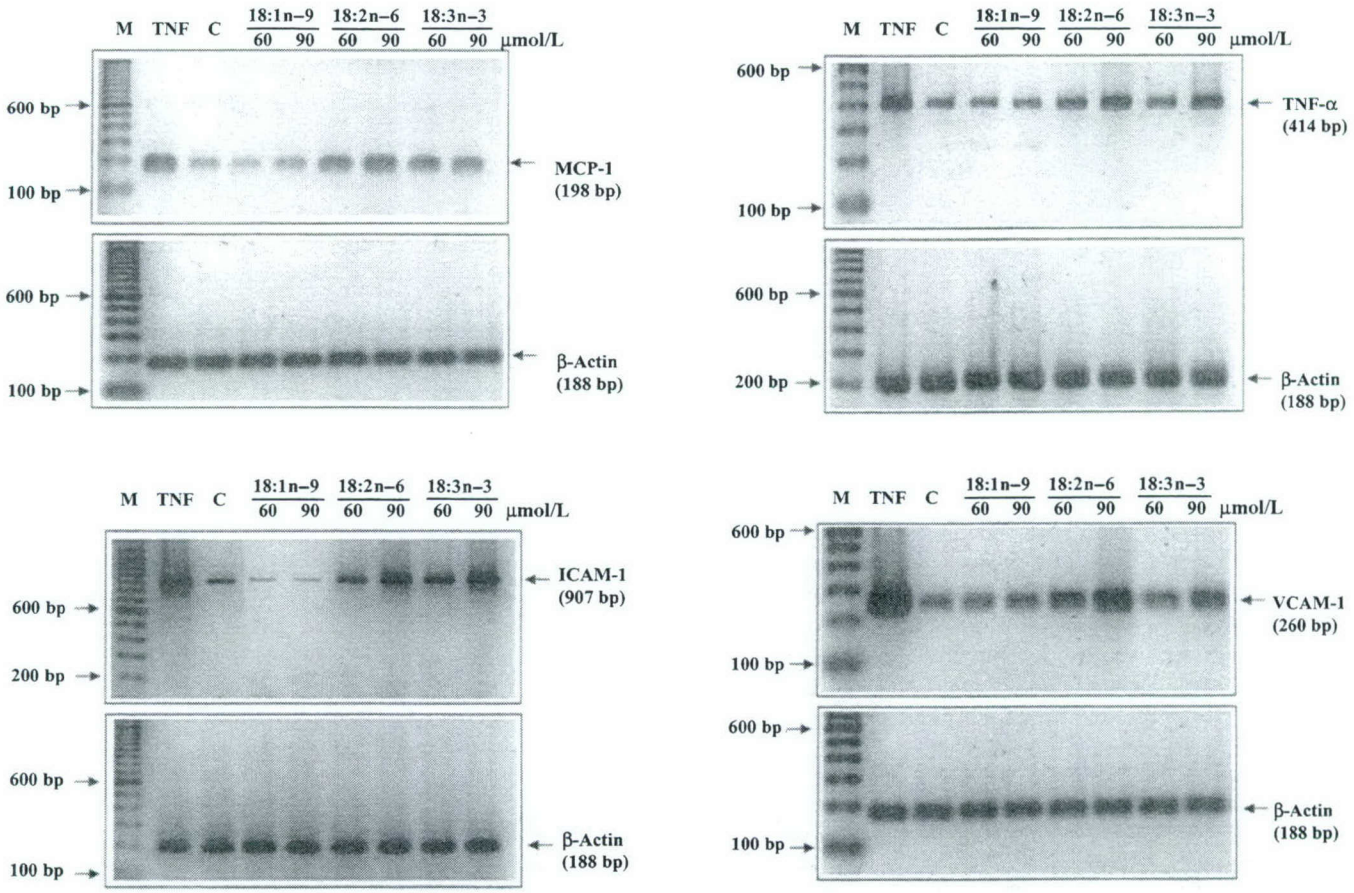

FIGURE 2. Effects of dietary fatty acids on monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNF-α), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) messenger RNA levels in human endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to specific unsaturated fatty acids for 3 h. β-Actin was used to indicate that the same amount of RNA was used per sample. The amplified PCR products were electrophoresed on a 2%–tris-borate EDTA agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR) and visualized by using phosphorimaging technology (FLA-2000; Fuji, Stamford, CT). bp, base pair; M, marker; C, control.

endothelial cells with oleic acid did not significantly affect luciferase activity in cells transfected with pNFκB-Luc. Compared with control cultures, linolenic acid exerted only a moderate effect on NF-κB transcriptional activation; however, treatment of transfected endothelial cells with linoleic acid resulted in a pronounced increase in luciferase activity, indicating a marked increase in transcriptional activation of NF-κB.

Similar results were observed in endothelial cells transfected with pAP1-Luc (Figure 1B). Among the fatty acids tested, linoleic acid stimulated AP-1 transcriptional activation most markedly compared with control cultures. In contrast, linolenic acid exerted more moderate effects, and oleic acid did not significantly affect luciferase expression.

Unsaturated fatty acids selectively induce the genes encoding for MCP-1 and TNF-α

The effects of treatment with selected unsaturated fatty acids on MCP-1 mRNA levels are shown in **Figure 2**. Among the tested fatty acids, linoleic acid at the concentration of 90 µmol/L stimulated the most pronounced induction of the MCP-1 gene (51 ± 1.97% above the control values as measured by the density of the fluorescent bands). Indeed, MCP-1 mRNA levels in endothelial cells treated with 90 µmol linoleic acid/L for 3 h were in the range observed in cells exposed to 20 µg TNF-α/L, which was used as a positive control. MCP-1 mRNA levels also increased in endothelial cells treated with 60 and 90 µmol linolenic acid/L (by 24 ± 2.46% and 30 ± 5.25%, respectively). In contrast, induction of the MCP-1 gene in endothelial cells exposed to oleic acid was approximately at the range observed in unstimulated endothelial cells.

The effects of treatment with selected unsaturated fatty acids on TNF-α mRNA levels are also shown in Figure 2. Similarly to the results for MCP-1 gene induction, treatment of HUVECs with linoleic acid markedly induced TNF-α mRNA levels (21 ± 3.22% above control values). In addition, linolenic acid at the dose of 90 µmol/L stimulated similar induction of the TNF-α gene. Independent of the dose used, treatment with oleic acid did not significantly affect TNF-α mRNA levels in cultured HUVECs.

Unsaturated fatty acids selectively induce the genes encoding for adhesion molecules

The effects of treatment with selected unsaturated fatty acids on ICAM-1 mRNA levels is shown in Figure 2. Exposure to both linoleic acid and linolenic acid induced similar dose-dependent increases in ICAM-1 mRNA levels. Specifically, linoleic and linolenic acids at the concentration of 90 µmol/L stimulated

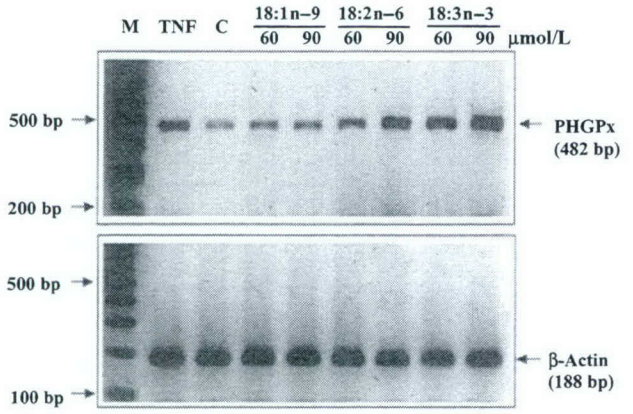

FIGURE 3. Effects of dietary fatty acids on phospholipid hydroperoxide glutathione peroxidase (PHGPx) mRNA levels in human endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to specific unsaturated fatty acids for 24 h. RT-PCR was performed as described in the legend to Figure 2. bp, base pair; M, marker; TNF, tumor necrosis factor α; C, control.

induction of the ICAM-1 gene by 32 ± 2.54% and 30 ± 3.34%, respectively. In contrast, exposure of HUVECs to oleic acid decreased ICAM-1 mRNA levels to ≈50% of control values.

The effects of specific unsaturated fatty acids on VCAM-1 mRNA levels in HUVEC are also shown in Figure 2. The most significant induction of the VCAM-1 gene (by 38 ± 2.20%) was observed in cells treated with 90 μmol linoleic acid/L. In addition, exposure to 90 μmol linolenic acid/L resulted in a slight increase in VCAM-1 mRNA levels (14 ± 1.88%). Treatment with oleic acid had no significant effect on VCAM-1 gene induction compared with control cultures.

Unsaturated fatty acids induce the gene encoding for PHGPx

The effects of selected fatty acids on PHGPx mRNA levels in HUVECs are shown in **Figure 3**. Compared with the control, treatment with oleic acid increased PHGPx mRNA levels by ≈30%. However, both linoleic and linolenic acids strongly, and dose dependently, stimulated induction of PHGPx gene in HUVECs. In fact, treatment with 60 and 90 μmol linoleic acid/L enhanced PHGPx mRNA levels by 60 ± 7.58% and 104 ± 5.04%, respectively. Furthermore, exposure to 60 and 90 μmol linolenic acid/L increased the induction of the PHGPx gene by 108 ± 6.48% and 121 ± 4.36%, respectively.

DISCUSSION

Mono- and polyunsaturated 18-carbon fatty acids provide a unique model for studying the cellular effects of fatty acids that differ in unsaturation independent of carbon length (9). In addition, the unsaturated fatty acids used in the present study are major dietary fatty acids. Endothelial cells were exposed to fatty acids at concentrations of 60 or 90 μmol/L, with an albumin concentration in the experimental media of 60 μmol/L. Normal plasma fatty acid concentrations can range from ≈90 to 1200 μmol/L; however, most fatty acids are bound to plasma components, mostly albumin (32, 33). In fact, the main factor in the availability of fatty acids for cellular uptake is determined by the ratio of fatty acids to albumin. Normally, this ratio can range from 0.15 to 4 under various conditions, with an average of ≈1 (32, 33). Thus, the experimental conditions used in the present study, which resulted in a ratio of fatty acids to albumin of 1 or 1.5, were within the physiologic range.

One of the most important functions of the vascular endothelium is to regulate inflammatory reactions (1). The development of inflammatory reactions is a normal defense mechanism in response to injury or activation of the vessel wall. The physiologic significance of such reactions is to maintain and repair the normal structure and function of the vessel wall. However, excessive inflammatory reactions with the development of a positive feedback inflammatory cycle can lead to severe tissue damage and are associated with vascular pathology, including the development of atherosclerotic plaques (34).

Induction of genes encoding for mediators of the inflammatory response, ie, inflammatory cytokines, chemokines, and adhesion molecules, can initiate leukocyte infiltration of the vessel wall. These mediators of the inflammatory response interact closely with each other in vivo. For example, ICAM-1 and VCAM-1 facilitate leukocyte adhesion to the vascular endothelium and both MCP-1 (35) and, to a lesser extent, TNF-α (36, 37) are potent chemoattractant factors that play a significant role in recruiting lymphocytes and monocytes into the vessel wall. In addition, TNF-α is a strong inducer of inflammatory reactions and can stimulate overexpression of MCP-1, inflammatory cytokines, and the adhesion molecules ICAM-1 and VCAM-1 (38). In fact, these strong proinflammatory properties of TNF-α were the reason that this cytokine was used as the positive control in our present study. In addition, the inflammatory genes examined in the present study, ie, those encoding for VCAM-1, ICAM-1, TNF-α, and MCP-1, are regulated by similar transcription factors, with dominant roles of NF-κB and AP-1 (22–25).

The importance of NF-κB and AP-1 in the induction of inflammatory reactions prompted us to study the effects of specific fatty acids on the transcriptional activity of these transcription factors in human endothelial cells. Among the unsaturated fatty acids studied, linoleic acid induced both NF-κB and AP-1 transcriptional activation most markedly. These data agree with our previous results in which our use of an electrophoretic mobility shift assay showed a marked activation of NF-κB (13) and AP-1 (39) in endothelial cells exposed to linoleic acid. It is possible that fatty acid–induced endothelial cell oxidative stress and disturbances in the glutathione redox status are responsible for the activation of these oxidative stress–responsive transcription factors. Intercellular glutathione is the major nonprotein thiol compound that regulates the cellular redox status. Depletion of glutathione concentrations and alterations in the equilibrium between the reduced and oxidized derivatives of glutathione can stimulate activation of NF-κB (40). To support this notion, we showed that exposure of endothelial cells to unsaturated fatty acids can result in a marked decrease in cellular glutathione concentrations and activation of NF-κB (9, 13). In addition, the glutathione precursor *N*-acetylcysteine prevented fatty acid–induced activation of NF-κB (41).

Glutathione peroxidases are a family of antioxidant enzymes that utilize glutathione in the reduction of hydrogen peroxide and alkyl hydroperoxides. Among the various glutathione peroxidases, PHGPx plays a unique role. In addition to reducing hydrogen peroxide and soluble hydroperoxides, PHGPx is the only antioxidant enzyme that can reduce hydroperoxy fatty acids that are integrated in cellular membranes (42) or lipoproteins (43).

PHGPx was also shown to be involved in silencing activities of cyclooxygenase or 5- and 15-lipoxygenases (44, 45), enzymes involved in the metabolism of unsaturated fatty acids. Results of the present study showed that exposure of endothelial cells to specific unsaturated fatty acids can markedly stimulate induction of PHGPx mRNA. In addition, the fatty acid–stimulated increases in PHGPx mRNA levels appeared to be correlated with the amount of unsaturated bonds in fatty acid molecules. For example, linolenic acid, followed by linoleic acid, enhanced induction of the PHGPx gene most markedly.

The present study provides compelling evidence that linoleic acid can induce profound inflammatory responses in cultured human endothelial cells. In fact, among all the unsaturated fatty acids studied, linoleic acid stimulated induction of inflammatory gene mRNA most markedly. Because expression of the inflammatory genes is regulated primarily by NF-κB and AP-1, a strong induction of NF-κB and AP-1 transcriptional activation by linoleic acid may explain the marked induction of the studied genes. In addition, not only linoleic acid but also specific oxidative products of this fatty acid can exert proinflammatory effects (46, 47). However, we observed that the lipoxygenase metabolites of linoleic acid, such as 13-hydroperoxyoctadecadienoic acid (13-HPODE) or 13-hydroxyoctadecadienoic acid (13-HODE), induce a different pattern of inflammatory responses in endothelial cells than does free linoleic acid. Specifically, exposure of HUVECs to 13-HPODE or 13-HODE does not induce the expression of VCAM-1 or E-selectin (48). In addition, polyunsaturated fatty acids, such as linoleic acid, can be nonenzymatically converted to 4-hydroxynonenal. However, exposure of HUVECs to 4-hydroxynonenal markedly stimulates apoptosis of vascular endothelial cells but does not result in activation of NF-κB or induction of adhesion molecules (49). Thus, even though linoleic acid can be converted to oxidized metabolites, it appears unlikely that 13-HPODE, 13-HODE, or 4-hydroxynonenal can contribute significantly to inflammatory reactions induced by this fatty acid. On the other hand, the effects of other metabolites of polyunsaturated fatty acids, eg, derivatives of the cytochrome P450 pathway, on inflammatory reactions in human endothelial cells remain to be determined. Our recent data suggest that epoxide metabolites of linoleic acid may have proinflammatory properties (50).

Although our data clearly indicate that specific unsaturated fatty acids can induce proinflammatory effects in endothelial cells, opposite results were reported when cells were exposed to selected n−3 or n−6 fatty acids for ≤72 h and coexposed to inflammatory cytokines, such as interleukin 1β (IL-1β) or TNF-α, for an additional 12 h. When such experimental approaches were used, preexposure to fatty acids inhibited cytokine-induced expression of inflammatory mediators, such as VCAM-1, on the surface of endothelial cells (51). Similar inhibition of ICAM-1 expression was also observed in cells pretreated with 13-HPODE before stimulation with IL-1β. However, simultaneous administration of 13-HPODE with IL-1β or TNF-α resulted in additive effects on ICAM-1 production (48). We showed that preexposure of endothelial cells to linoleic acid can cross-amplify TNF-α–mediated induction of cellular oxidative stress and endothelial cell dysfunction (13) but does not potentiate or even inhibit NF-κB–dependent transcription (13, 41). To explain this phenomenon, it was proposed that fatty acid–induced activation of NF-κB could lead to increased numbers of NF-κB inhibitory subunits, which, in turn, could prevent further activation of this transcription factor in cells exposed to cytokines at later time points (13).

In contrast with linoleic and linolenic acids, which exerted strong or moderate proinflammatory responses, respectively, oleic acid diminished inflammatory gene mRNA levels in endothelial cells. These data agree with previous reports on antioxidant effects mediated by oleic acid. For example, a diet enriched in oleic acid markedly decreases LDL susceptibility to oxidation and LDL-protein modification in mildly hypercholesterolemic patients (10). Similar results were obtained in experimental animals fed a diet enriched in oleic acid (52). Extensive evidence also indicates the protective and antioxidant effects of oleic acid on endothelial cell activation. Cellular treatment with this fatty acid protects endothelial cells against cytokine-induced VCAM-1, ICAM-1, or E-selectin overexpression (53). In addition, supplementation with oleic acid protects endothelial cells against hydrogen peroxide–induced cytotoxicity (16) and against dysfunction of the endothelial barrier as mediated by oxidized LDL (54).

In conclusion, the present study showed that specific unsaturated dietary fatty acids can induce highly individual effects on endothelial cell activation and contribute differently to induction of the inflammatory genes in vascular endothelial cells. Among the fatty acids studied, linoleic acid stimulated inflammatory gene mRNA most markedly. In contrast, oleic acid appeared to silence the induction of various proinflammatory genes in endothelial cells. These results showed that specific unsaturated dietary fatty acids, such as linoleic acid and to a lesser extent linolenic acid, can stimulate the development of proinflammatory environments within the vascular endothelium.

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Linoleic Acid-Induced VCAM-1 Expression in Human Microvascular Endothelial Cells Is Mediated by the NF-κB-Dependent Pathway

Hyen Joo Park, Yong Woo Lee, Bernhard Hennig, and Michal Toborek

Abstract: *Vascular cell adhesion molecule-1 (VCAM-1) has been reported to play an important role in cancer metastasis via the adhesive interaction between tumor cells and endothelial cells. In this study, we examined the effects of linoleic acid on VCAM-1 expression and its transcriptional regulatory mechanism in human microvascular endothelial cells (HMEC-1). Time- and dose-dependent increases of VCAM-1 mRNA levels were observed in linoleic acid-treated HMEC-1 as detected by reverse transcriptase-polymerase chain reaction. Flow cytometry analysis showed a significant and dose-dependent upregulation of VCAM-1 expression in HMEC-1 stimulated with linoleic acid compared with controls. To clarify the transcriptional regulatory pathway, we investigated the role of nuclear factor-κB (NF-κB) in the expression of VCAM-1 by linoleic acid in HMEC-1. Nuclear extracts from HMEC-1 stimulated with linoleic acid showed a dose-dependent increase in binding activity to the NF-κB consensus sequences. These effects were preventable by cotreatment with inhibitors of NF-κB activity, such as sodium salicylate, aspirin, or pyrrolidine dithiocarbamate. In addition, pretreatment with NF-κB inhibitors markedly suppressed the ability of linoleic acid to induce VCAM-1 gene expression. The role of NF-κB in linoleic acid-induced VCAM-1 expression was confirmed by functional promoter studies in HMEC-1 transfected with reporter constructs of the VCAM-1 promoter with or without mutated NF-κB binding site. These results indicate that linoleic acid upregulates VCAM-1 expression in HMEC-1 through the NF-κB-dependent pathway.*

Introduction

Dietary fat is considered to be one of the main risk factors of carcinogenesis. For example, a positive correlation was reported between dietary fat intake and increased risks for the development of breast, colon, and prostate cancers (1–3). However, the role of dietary fat in the development of human breast cancer has recently been questioned. Although data obtained from animal studies (reviewed in Ref. 4), international correlation analyses (5,6), and meta-analysis of dietary fat intervention studies (7) strongly indicate the association between fat consumption and the development of breast cancer, data from prospective cohort studies on dietary fat and breast cancer (8–10) suggested that dietary fat might not be a risk factor for human breast cancer. Among different dietary fatty acids, it appears that linoleic acid (C18:2, n-6) can promote carcinogenesis (11–14). In addition to its role in carcinogenesis, dietary linoleic acid can also enhance the metastatic formation of mammary tumors. For example, a linoleic acid-enriched diet increased the rate of metastasis of mammary cancer to the lung in rats (15).

The formation of blood-borne metastasis is a complex process that requires several steps. However, a growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical event in metastasis formation (16,17). This process requires the binding of tumor cells to specific adhesion molecules on the surface of endothelial cells followed by migration of tumor cells through the endothelium into underlying tissues (16). Evidence indicates that, among several adhesion molecules that can be involved in this process, vascular cell adhesion molecule-1 (VCAM-1) may play one of the most important roles. For example, it was demonstrated that VCAM-1 facilitated adhesion of metastatic breast tumor cells to endothelial cells stimulated by shear stress (18). In patients with breast cancer or gastric cancers, serum levels of soluble VCAM-1 were closely correlated with disease progression (19,20). Upregulation of VCAM-1 was also shown to be involved in adhesion of RAW117 lymphoma cells (21) or melanoma cells to hepatic sinusoidal endothelial cells (22). In addition, evidence indicated the role of VCAM-1 in adhesion of B9/BM1 cells to bone marrow-derived endothelial cells (23) and adhesion of DU145 cells (the cell line derived from cerebral metastasis of prostate carcinoma) to human brain microvascular endothelial cells (24).

VCAM-1 is a 110-kDa member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion protein (25). It facilitates tumor cell adhesion through binding of an integrin counterreceptor, very

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late antigen-4 (26). Functional studies on the activity of the VCAM-1 gene promoter have shown that regulation of VCAM-1 gene expression in endothelial cells appears to be complex and related to the type of stimuli. For example, VCAM-1 induction by inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor-α (TNF-α), as well as by lipopolysaccharide (LPS), strongly relies on activation of nuclear transcription factor-κB (NF-κB) (27,28). In contrast, recent evidence indicated that IL-4-induced VCAM-1 expression is independent of NF-κB activation (29,30). Thus the specific role of NF-κB in linoleic acid-induced overexpression of the VCAM-1 gene is uncertain and was chosen as the subject of the present study.

Because of the significance of dietary linoleic acid and VCAM-1 expression in cancer metastasis, the aim of the present study was to investigate the molecular signaling pathways involved in linoleic acid-induced VCAM-1 up-regulation in human microvascular endothelial cells. We have determined that linoleic acid can induce VCAM-1 expression at the mRNA and protein levels. Furthermore, we provide evidence that linoleic acid-stimulated expression of the VCAM-1 gene is mediated by activation of NF-κB.

Materials and Methods

Cell Culture

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center). HMEC-1 were cultured in MCDB 131 medium (Sigma, St. Louis, MO) enriched with 10% fetal bovine serum, 1% penicillin-streptomycin, 1 μg/ml hydrocortisone, and 0.01 μg/ml epidermal growth factor in a 5% CO_2 atmosphere at 37°C. Linoleic acid (>99% pure; Nu-Chek Prep, Elysian, MN) was added to the medium as described previously (31). In the present study, linoleic acid was used at ≤50 μM, i.e., levels that do not induce cytotoxic effects in vascular endothelial cells (32).

In selected experiments, HMEC-1 were pretreated for 1 h with salicylates (aspirin or sodium salicylate) or for 30 min with pyrrolidine dithiocarbamate (PDTC). Salicylates were used at ≤10 mM and PDTC at ≤25 μM.

Electrophoretic Mobility Shift Assay

Nuclear extracts from HMEC-1 were prepared according to the method of Beg et al. (33). Binding reactions were performed in a 20-μl volume containing 4–10 μg of nuclear protein extracts, 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μg of poly(dI-dC) (nonspecific competitor), and 40,000 cpm of ^{32}P-labeled specific oligonucleotides that contained the consensus sequence for NF-κB site (5′-AGTTGAGGGGACTTTCCCAGG-3′). The resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using 0.25× TBE buffer (50 mM Tris·Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were employed in supershift experiments.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted by the use of TRIreagent (Sigma) and reverse-transcribed at 42°C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris·HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 U/μl of recombinant RNasin ribonuclease inhibitor, 15 U/μg of avian myeloblastosis virus reverse transcriptase (RT), and 0.5 μg of oligo$(dT)_{15}$ primer. For amplification of VCAM-1 and of β-actin (a housekeeping gene), the following primer combinations were used: 5′-ATGACATGCTTGAGCCAGG-3′ and 5′-GTGTCTCCTTCTTTGACACT-3′ (VCAM-1; expecting 260-bp fragment) (34) and 5′-AGCACAATGAAGATCAAGAT-3′ and 5′-TGTAACGCAACTAAGTCATA-3′ (β-actin; expecting 188-bp fragment) (35). The polymerase chain reaction (PCR) mixture consisted of a *Taq* PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the RT reaction, and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated 20 times. Amplification was linear within the range of 15–30 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

Flow Cytometry for VCAM-1 Determination

VCAM-1 protein expression was determined by flow cytometry. Briefly, HMEC-1 were grown to confluence on six-well culture plates and treated with linoleic acid for 12 h. HMEC-1 were then washed with Hanks' buffer and gently harvested by trypsin-EDTA. Cells were washed twice with phosphate-buffered saline (PBS) and incubated for 1 h on ice with saturating amounts of monoclonal mouse anti-human VCAM-1 antibody labeled with fluorescein isothiocyanate (R & D Systems, Minneapolis, MN). Fluorescein isothiocyanate-labeled mouse anti-human IgG1 was used as the isotype control (R & D Systems). After incubation with antibodies, samples were washed twice with ice-cold PBS and analyzed with 10,000 cells/sample in a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). After correction for unspecific binding (isotype control), specific mean fluorescence intensity was expressed as the indicator of VCAM-1 protein expression.

Transient Transfection and Reporter Gene Assays

Transient transfections of HMEC-1 were performed using pFx-7 (Invitrogen, Carlsbad, CA) as described earlier (36). Cells were transfected with 10 μg of the VCAM-1 pro-

moter constructs with or without mutated NF-κB site (pF3-mNF-κB-CAT3 and pF3-CAT3, respectively) and cotransfected with 4 μg of the pGL3-Luc control vector (Promega, Madison, WI) to normalize transfection rates. The reporter constructs, pF3-mNF-κB-CAT3 and pF3-CAT3, were kind gifts from Dr. Andrew S. Neish (Emory University School of Medicine). Generation of these constructs was described and characterized earlier (27,37). After transfection, cultures were maintained in normal growth medium for 24 h and then exposed to 50 μM linoleic acid for an additional 24 h in MCDB 131 medium enriched with 5% fetal bovine serum. After treatment exposure, cells were washed twice with PBS and lysed in 100 μl of reporter lysis buffer (Promega). Chloramphenicol acetyltransferase (CAT) activity was determined using the method of Gorman et al. (38). The cell lysates, normalized for protein levels, were incubated for 4 h at 37°C with a reaction mixture composed of 125 mM Tris·HCl (pH 7.8), 0.83 mM acetyl coenzyme A, and 3 μl of [^{14}C]chloramphenicol (25 μCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ). Then acetylated and nonacetylated forms of chloramphenicol were extracted with ethyl acetate and separated by thin-layer chromatography using the solvent system with chloroform-methanol (95:5, vol/vol). After autoradiography, the zones corresponding to acetylated or nonacetylated chloramphenicol were cut from the plates, and radioactivity was counted in a liquid scintillation counter for quantitation of CAT activity. The CAT activity was normalized according to luciferase activity, which was determined using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

Statistical Analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS, Chicago, IL). One-way analysis of variance was used to compare mean responses among the treatments. The treatment means were compared using Bonferroni's least significant difference procedure. $P < 0.05$ was considered significant.

Results

Linoleic Acid Activates NF-κB in HMEC-1

To determine whether linoleic acid can activate NF-κB in HMEC-1, cells were exposed to this fatty acid for 2 h, and NF-κB binding was analyzed by electrophoretic mobility shift assay (EMSA) using nuclear extracts of the treated cells. Results of these experiments are shown in Fig. 1, A and B. Figure 1A depicts the effects of linoleic acid on the binding activity of NF-κB in HMEC-1. A slight endogenous activity of NF-κB was observed in control cultures (Fig. 1A, Lane 2). However, when the cells were stimulated with 50 μM linoleic acid, a marked increase in NF-κB binding activity was detected. This binding was specifically inhibited by an unlabeled competitor DNA containing the consensus NF-κB sequence (Fig. 1A, Lane 7). In addition, the identity of NF-κB binding was confirmed by supershift experiments with antibodies against specific NF-κB subunits, p50 and p65 (Fig. 1B, Lanes 3 and 4).

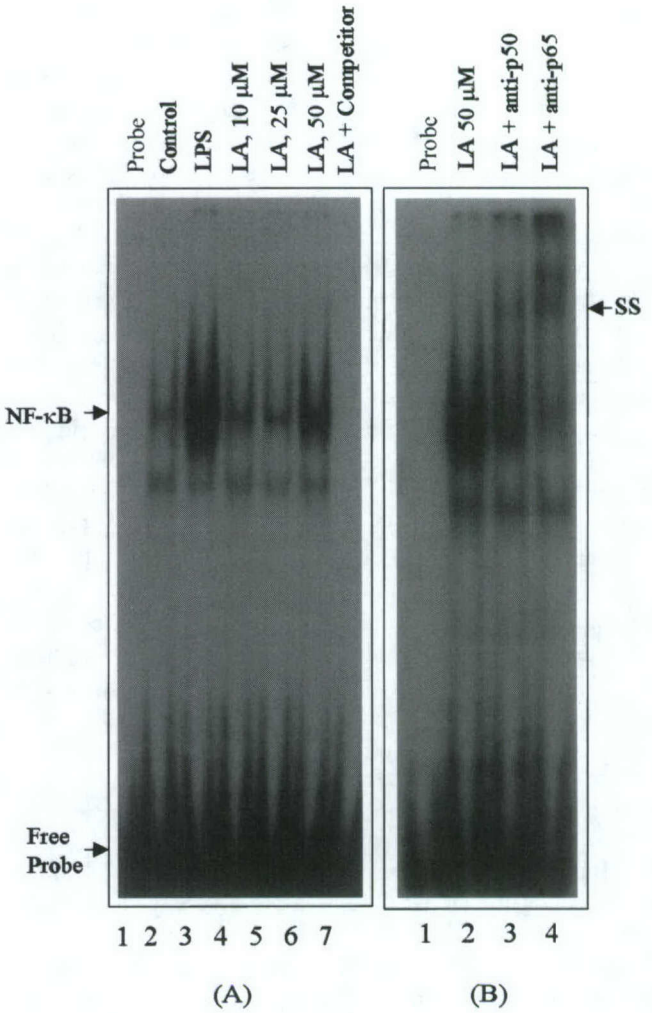


Figure 1. A: treatment with 50 μM linoleic acid (LA) enhances nuclear factor-κB (NF-κB) binding in human microvascular endothelial cells (HMEC-1) as analyzed by electrophoretic mobility shift assay. HMEC-1 were untreated (Lane 2) or treated for 2 h with increasing doses of linoleic acid (Lanes 4–6). Competition study was performed by addition of excess unlabeled oligonucleotide (Lane 7) using nuclear extracts from cells treated with 50 μM linoleic acid. Lane 1, probe alone; Lane 3, lipopolysaccharide (LPS, 1 μg/ml, positive control). B: supershift analysis of LA-induced NF-κB binding activity in HMEC-1. Nuclear extracts were prepared from cells treated with 50 μM linoleic acid for 2 h (Lanes 2–4) and incubated with anti-p50 antibody (Lane 3) or anti-p65 antibody (Lane 4) for 25 min before addition of ^{32}P-labeled probe. Lane 1, probe alone. SS, bands shifted by specific antibodies.

To further study linoleic acid-induced activation of NF-κB, HMEC-1 were pretreated for 1 h with aspirin or sodium salicylate or for 30 min with PDTC before a coexposure to linoleic acid for 2 h. Both salicylates and PDTC are widely used as inhibitors of NF-κB activation. Pretreatment with aspirin (Fig. 2A), sodium salicylate (Fig. 2B), or PDTC (Fig. 2C) resulted in dose-dependent inhibitions of NF-κB activation in HMEC-1 exposed to linoleic acid.

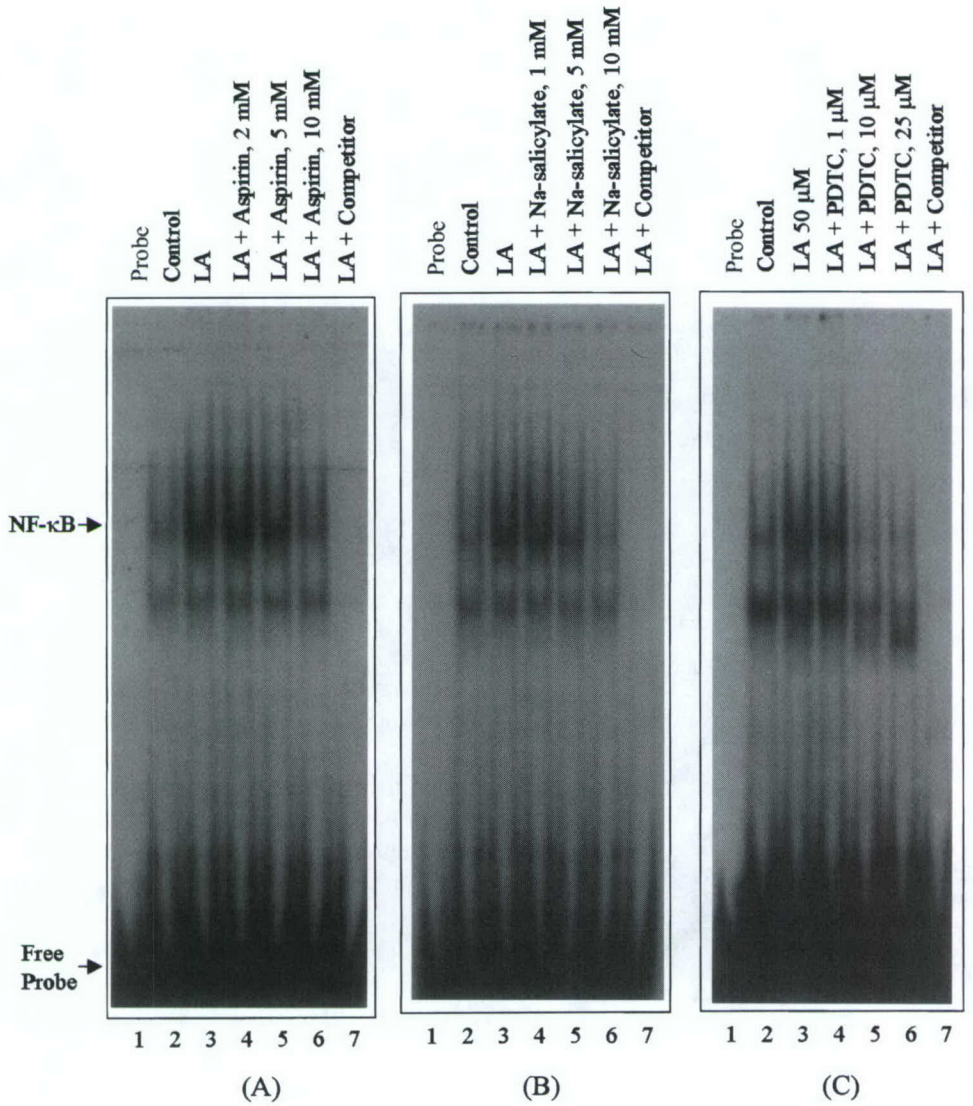


Figure 2. Pretreatment with aspirin, sodium salicylate, or pyrrolidine dithiocarbamate (PDTC) blocks linoleic acid-induced NF-κB DNA-binding activity in HMEC-1 as measured by electrophoretic mobility shift assay. HMEC-1 were pretreated for 1 h with indicated concentrations of aspirin (A) or sodium salicylate (B) or for 30 min with PDTC (C) before 2 h of treatment with 50 μM linoleic acid (Lanes 4–6). Lane 1, probe alone; Lane 3, 50 μM linoleic acid alone; Lane 7, competition study performed by addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 50 μM linoleic acid.

Linoleic Acid Induces VCAM-1 Expression in HMEC-1

To investigate whether exposure to linoleic acid can induce expression of VCAM-1 in microvascular endothelial cells, HMEC-1 were treated with 50 μM linoleic acid for increasing time periods, and the VCAM-1 mRNA level was determined by semiquantitative RT-PCR. As shown in Fig. 3, low levels of constitutively expressed VCAM-1 mRNA were detected in control cells (no linoleic acid supplementation). On the other hand, mRNA transcripts for VCAM-1 were clearly increased in linoleic acid-treated cells. Upregulation of VCAM-1 expression was observed after 1 h of exposure to linoleic acid, reaching maximal levels at 4 h (Fig. 3A).

Figure 3B shows that 4 h of exposure of HMEC-1 to linoleic acid resulted in a dose-dependent increase in the VCAM-1 mRNA. The most marked VCAM-1 expression was observed in HMEC-1 cultures treated with 50 μM linoleic acid. Additional increase in linoleic acid concentration did not further potentiate VCAM-1 expression (data not shown).

Figure 4 indicates the effects of increasing concentrations of linoleic acid treatment on VCAM-1 protein expression as measured by flow cytometry. In agreement with RT-PCR data, VCAM-1 protein was constitutively expressed in untreated HMEC-1. However, in cells treated with linoleic acid for 12 h, expression of this adhesion molecule was markedly upregulated in a dose-dependent manner.

Linoleic Acid-Induced VCAM-1 Expression in HMEC-1 Is Mediated by NF-κB

To determine whether linoleic acid-mediated activation of NF-κB is involved in upregulation of VCAM-1, expression of the VCAM-1 gene was studied in HMEC-1 pre-

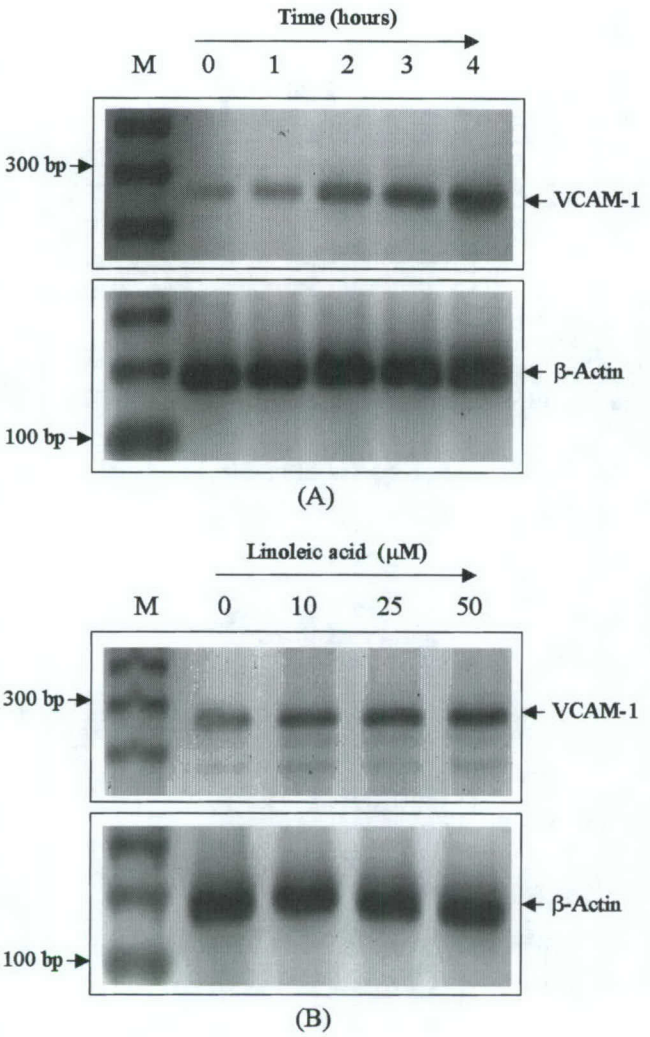


Figure 3. Time- and dose-dependent upregulation of vascular cell adhesion molecule-1 (VCAM-1) mRNA expression in HMEC-1 induced by linoleic acid as measured by reverse transcriptase-polymerase chain reaction (RT-PCR). HMEC-1 were exposed to 50 μM linoleic acid for indicated period of time (A) or treated with increasing concentrations of linoleic acid for 4 h (B). PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. Predicted sizes of RT-PCR products for VCAM-1 and β-actin are 260 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder).

treated with different doses of NF-κB inhibitors and exposed to 50 μM linoleic acid for 4 h. Similar to our studies presented in Fig. 2, aspirin, sodium salicylate, and PDTC were employed to inhibit NF-κB. Effects of these NF-κB inhibitors on linoleic acid-induced overexpression of the VCAM-1 gene are reflected in Fig. 5. As indicated, 1 h of pretreatment with increasing doses of aspirin (Fig. 5A) or sodium salicylate (Fig. 5B), as well as 30 min of treatment with PDTC (Fig. 5C), markedly and in a dose-dependent manner decreased linoleic acid-mediated stimulation of the VCAM-1 gene.

To further determine that the NF-κB binding site plays the critical role in linoleic acid-induced VCAM-1 gene expression in HMEC-1, cells were transfected with the construct of normal VCAM-1 promoter (pF3-CAT3) or with a

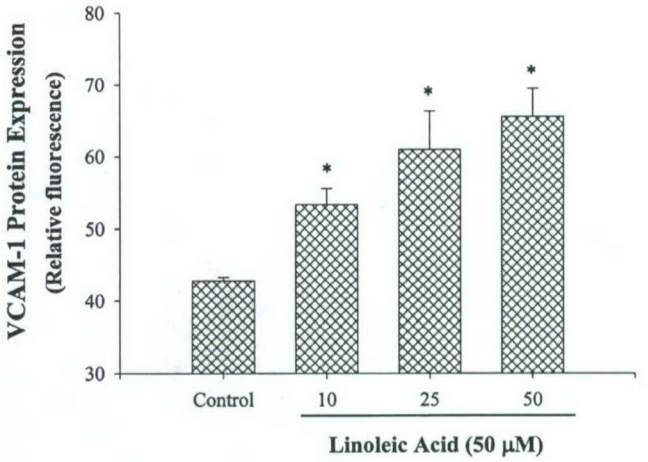
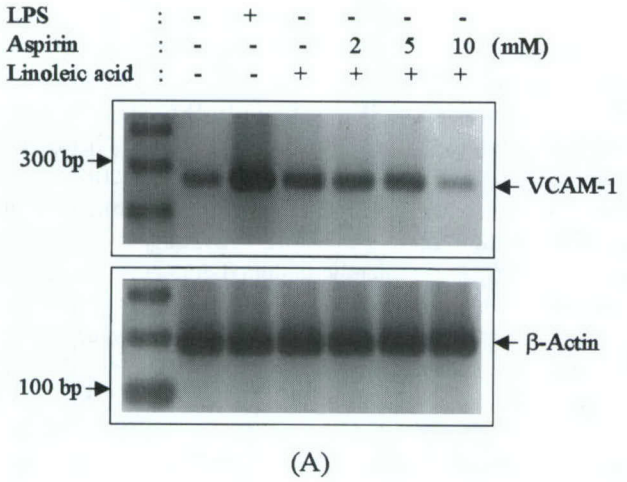


Figure 4. Linoleic acid increases VCAM-1 protein expression in HMEC-1 as measured by flow cytometry. HMEC-1 were exposed to increasing concentrations of linoleic acid for 12 h. Values are means ± SD expressed as relative fluorescence intensity and corrected for unspecific binding. *, Significantly different from untreated control ($P < 0.05$).

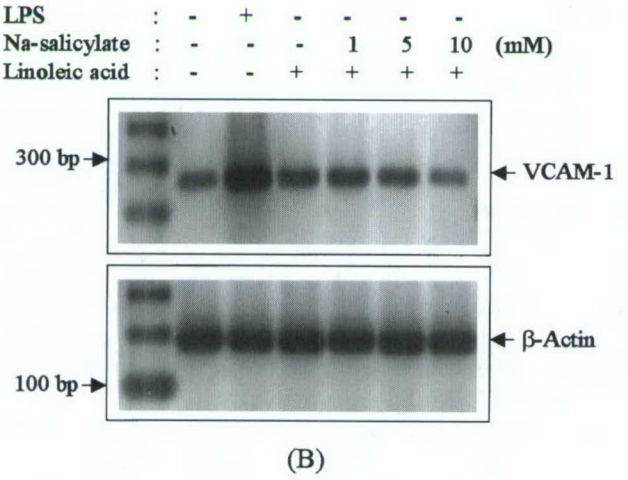
similar construct that had a mutated NF-κB binding site (pF3-mNF-κB-CAT3). As indicated in Fig. 6, exposure to linoleic acid induced CAT activity only in cells transfected with the pF3-CAT3 construct. In contrast, mutation of the NF-κB site completely inhibited linoleic acid-induced stimulation of CAT activity in HMEC-1 transfected with the pF3-mNF-κB-CAT3 construct.

Discussion

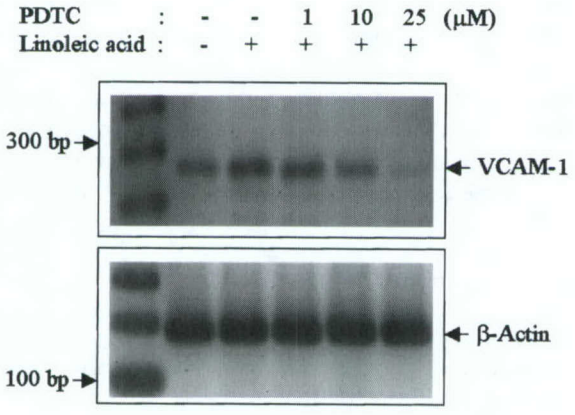
Adhesive interactions between vascular endothelial cells and tumor cells play a critical role in the process of metastatic tumor dissemination. This process is mediated by adhesion molecules, which are expressed on the surface of endothelial cells, and specific integrin receptors, which are present on tumor cells. After adhesion, tumor cells can migrate across the vascular endothelium and establish new metastatic colonies. In addition, this process protects tumor cells against destruction by cells of the immune system (17). Thus, although adhesion molecules do not directly influence carcinogenesis, they can markedly stimulate blood-borne tumor metastasis. It appears that, among different adhesion molecules involved in endothelial cell-tumor cell interactions, VCAM-1 may play one of the most important roles (19–24). Furthermore, determination of VCAM-1 expression can serve as an important marker in cancer diagnosis. It is well known that angiogenesis, i.e., the formation of new capillaries from preexisting blood vessels, is essential for tumor growth and metastasis (39). Because adhesion molecules, including VCAM-1, are expressed on the surface of newly formed vascular endothelium, their elevated levels can indicate an active angiogenesis (19). Thus overexpression of VCAM-1 can have two distinctive features in cancer biology and diagnosis: 1) it can stimulate metastasis through facilitation of tumor cell adherence to the vascular endothe-



(A)



(B)



(C)

Figure 5. Pretreatment with aspirin, sodium salicylate, or PDTC impedes induction of VCAM-1 mRNA expression in linoleic acid-treated HMEC-1. Cells were pretreated for 1 h with indicated concentrations of aspirin (A) or sodium salicylate (B) or for 30 min with PDTC (C) before 4 h of treatment with 50 μM linoleic acid and assayed for VCAM-1 mRNA expression by RT-PCR. LPS (1 μg/ml) was used as positive control.

lium, and 2) it can serve as a marker of angiogenesis, which is associated with tumor growth.

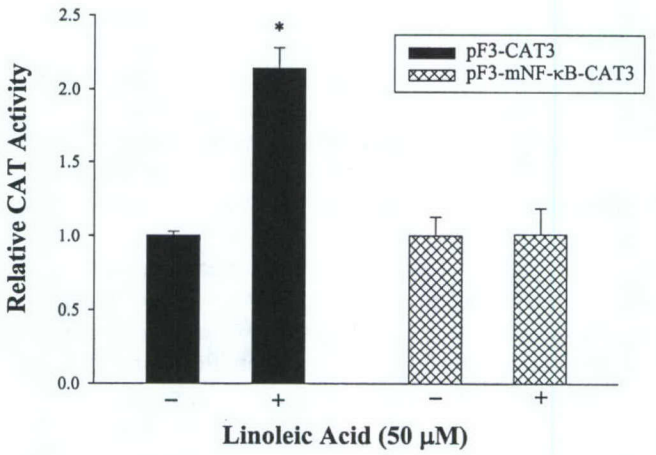


Figure 6. Functional analysis of NF-κB binding site of the human VCAM-1 promoter in linoleic acid-treated HMEC-1. Cells were transfected with pF3-CAT3 or pF3-mNF-κB-CAT3 construct and untreated or treated with 50 μM linoleic acid for 24 h. Mutation of NF-κB site in VCAM-1 promoter construct completely inhibited linoleic acid-induced chloramphenicol transferase (CAT) activity. *, Significantly different from untreated control ($P < 0.05$).

A number of clinical and animal studies have implicated the intake and composition of dietary fats in expression of endothelial cell adhesion molecules, including upregulation of VCAM-1 (40–43). In the average American diet, fat accounts for 35–40% of energy (44), and such overconsumption of foods rich in fat may be a major risk for cancer development and metastasis. Although the role of dietary fat in human breast cancer has been recently questioned (8–10), dietary factors, including excessive intake of fat, are considered to contribute to 35% of all cancers (1). Part of the carcinogenic and prometastatic effects of dietary fat can be related to modulation of the functions of the vascular endothelium. It appears that, among different dietary fatty acids, linoleic acid can alter endothelial cell metabolism most significantly (45) and, thus, induce the development of cancer metastasis (12,13,15). Because it is an unsaturated fatty acid, linoleic acid can undergo peroxidative pathways initiated by hydrogen abstraction followed by oxygen attack on the generated lipid alkyl radical (46). Several reports suggest that linoleic acid can act as a potent prooxidant in endothelial cells in culture. For example, linoleic acid can enhance radical adduct formation in endothelial cells exposed to iron-induced oxidative stress (47), decrease glutathione levels (31), and increase peroxisomal β-peroxidation (48), a pathway that leads to the production of hydrogen peroxide. Degradation of linoleic acid via peroxidative pathways also can lead to formation of highly cytotoxic products, such as linoleic acid hydroperoxides or 4-hydroxy-2-(E)-nonenal (49). Metabolism of polyunsaturated fatty acids through lipoxygenase-mediated processes also may play an important role in cancer biology. For example, it was shown that 12(*S*)-hydroxyeicosatetraenoic acid, a metabolite of arachidonic acid generated in the reaction catalyzed by 12-lipoxygenase, can influence angiogenesis and formation of cancer metastasis (50). However, endothelial cell effects of linoleic acid have been primarily studied in

cells isolated from major vascular vessels, such as pulmonary artery (31,32,51) or umbilical veins (52). It is well known that the structure and functions of endothelial cells that originated from different tissues and vessels can differ markedly (53). Therefore, the present study focused on mechanistic effects of linoleic acid on induction of VCAM-1 in human microvascular endothelial cells, i.e., the cell type that is most relevant to cancer metastasis.

In the present study we report that treatment of HMEC-1 with linoleic acid results in an increase of the steady-state concentration of the VCAM-1 mRNA in a time- and dose-dependent manner (Fig. 3). In addition, flow cytometry analysis showed that linoleic acid-induced upregulation of the VCAM-1 gene is correlated with a significant and dose-dependent overexpression of VCAM-1 protein in HMEC-1 (Fig. 4). These results are in agreement with earlier reports that indicated upregulation of another adhesion molecule, such as intercellular adhesion molecule-1, in endothelial cells treated with linoleic acid (52). Recent evidence also indicated that an oxidized derivative of linoleic acid, 13-hydroperoxyoctadecadienoic acid, can induce VCAM-1 gene expression in endothelial cells (54). On the other hand, a 72-h preexposure of endothelial cells to selected n-3 or n-6 fatty acids, followed by a cotreatment with IL-1β or TNF-α for an additional 12 h, resulted in an inhibition of cytokine-induced VCAM-1 expression compared with cells that were not pretreated with fatty acids (55). However, a very different experimental setting used in that study was, most likely, responsible for this discrepancy with our present results.

The current study also reveals that treatment of HMEC-1 with linoleic acid can activate NF-κB. These results are in agreement with earlier reports on NF-κB activation by linoleic acid in porcine pulmonary artery endothelial cells (51, 56). It is possible that linoleic acid-mediated induction of oxidative stress (51), a decrease in cellular glutathione (31), and alterations of cellular redox status (31,51) are responsible for activation of NF-κB. To support the role of oxidative stress in linoleic acid-induced activation of NF-κB, this effect was attenuated by salicylates and PDTC (Fig. 2). Aspirin and sodium salicylate have been shown to specifically inhibit the activation of NF-κB by preventing the degradation of IκB, an NF-κB inhibitory subunit, and blocking the translocation of NF-κB into the nuclear compartment (57, 58). PDTC, the radical-scavenging thiol compound, is also widely used as an inhibitor of NF-κB activation (59,60).

NF-κB binding sites are located in the promoter regions of the genes encoding for adhesion molecules, including VCAM-1 (61). Two adjacent κB sites located at positions –77 and –63 relative to the transcription start site were identified in the VCAM-1 promoter (27,28). The role of these κB binding sites in the induction of the VCAM-1 gene is not fully understood and may depend on the type of stimulus. For example, NF-κB binding appears to be critical in TNF-α or lipopolysaccharide-induced VCAM-1 expression (27,28,61). In contrast, IL-4-mediated induction of the VCAM-1 gene is independent of NF-κB activation. This phenomenon was reported in endothelial cells (29) and in other types of vascular cells (30). These conflicting reports on the role of NF-κB activation in VCAM-1 gene expression prompted us to investigate the role of this transcription factor in linoleic acid-mediated stimulation of VCAM-1 in HMEC-1. In the present study, two different lines of experiments proved that linoleic acid-induced activation of NF-κB and induction of the VCAM-1 gene are interrelated. First, pretreatment of the HMEC-1 with inhibitors of NF-κB activation, such as salicylates or PDTC, completely inhibited linoleic acid-induced VCAM-1 expression (Fig. 5). Second, reporter gene assays were performed using normal VCAM-1 promoter reporter construct as well as similar construct but with mutated NF-κB binding site. As indicated in Fig. 6, mutation of the NF-κB binding site in the VCAM-1 promoter region completely abolished linoleic acid-induced expression of the reporter gene. These results specifically indicate the importance of NF-κB activation in linoleic acid-induced expression of the VCAM-1 gene. On the other hand, low doses of linoleic acid, such as 10 or 25 μM, stimulated VCAM-1 expression (Figs. 3 and 4) but were not sufficient to activate NF-κB. To explain this discrepancy, it should be pointed out that the promoter region of the VCAM-1 gene contains binding sites not only for NF-κB, but also for several other transcription factors, such as AP-1, SP-1, GATA-1, or Ets (27,28). It is possible that, in low concentrations of linoleic acid, these other transcription factors may participate in induction of the VCAM-1 gene. In contrast, in higher concentrations of linoleic acid, such as 50 μM, it appears that activation of NF-κB is the critical factor in induction of VCAM-1 expression.

In conclusion, our studies have demonstrated that linoleic acid induces VCAM-1 expression in HMEC-1 through the activation of NF-κB. More importantly, because the ability of cancer cells to interact with the endothelium appears to be a prerequisite for the potential of distant metastasis and because VCAM-1 is considered to be a crucial adhesion molecule in this process, the present study may have significant therapeutic implications in developing a novel strategy against cancer metastasis. Finally, these studies provide a mechanistic insight into the role of specific dietary lipids in metastasis.

Acknowledgments and Notes

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Linoleic acid induces MCP-1 gene expression in human microvascular endothelial cells through an oxidative mechanism

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Abstract

Linoleic acid is a dietary fatty acid that appears to play an important role in activation of the vascular endothelium under a variety of pathological conditions, including development of atherosclerosis or cancer metastasis. Evidence indicates that inflammatory responses may be an underlying cause of endothelial cell pathology induced by linoleic acid. However, the profile of inflammatory mediators and the potential mechanisms involved in inflammatory reactions stimulated by the exposure to linoleic acid are not fully understood. The present study focused on the mechanisms of linoleic acid-induced expression of monocyte chemoattractant protein-1 (MCP-1) gene in human microvascular endothelial cells (HMEC-1). Treatment of HMEC-1 with increasing doses of linoleic acid markedly activated an oxidative stress-responsive transcription factor, nuclear factor-κB (NF-κB). In addition, exposure to linoleic acid induced a time- and concentration-dependent overexpression of the MCP-1 gene. Increased MCP-1 mRNA levels were observed in HMEC-1 treated with linoleic acid at doses as low as 10 μM. Linoleic acid-induced overexpression of the MCP-1 gene was associated with a significant elevation of MCP-1 protein levels. Most importantly, preexposure of HMEC-1 to antioxidants, such as pyrrolidine dithiocarbamate (PDTC) or N-acetylcysteine (NAC), attenuated linoleic acid-induced MCP-1 mRNA expression. The obtained results indicate that linoleic acid triggers MCP-1 gene expression in human microvascular endothelial cells through oxidative stress/redox-related mechanisms.

Keywords: dietary fatty acids; vascular endothelium; cancer metastasis; atherosclerosis; oxidative stress

1. Introduction

Induction of inflammatory genes plays an important role in the physiological and pathological functions of the vascular endothelium. For example, the overexpression of adhesion molecules on the surface of endothelial cells may stimulate adhesion and migration of both tumor cells or monocytes/macrophages across the vascular endothelium [1]. In addition, increased expression of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) may play a critical role in the biology of vascular dysfunction. A member of the CC chemokine family, human MCP-1 stimulates chemotaxis and transmigration of monocytes, lymphocytes, and granulocytes [2]. Increased production of MCP-1 may be involved in a variety of processes, including early phases of atherosclerosis [3,4] and cancer metastasis [5–7].

There are at least two distinct mechanisms by which MCP-1 may participate in cancer metastasis: MCP-1 may induce the unidirectorial migration of inflammatory cells [2]. MCP-1 may be chemotactic to tumor cells [5]. This latter effect was demonstrated using MCF-7 cells, a cell line obtained from human breast carcinoma [5]. The chemotactic influence of MCP-1 on tumor cells was shown to be mediated by a receptor-stimulated signaling pathway [8]. Thus, it appears that MCP-1 can directly attract tumor cells and induce tumor cell migration across the vascular endothelium with the subsequent generation of tumor metastasis. In addition to such direct effects, chemotactic properties of MCP-1 towards leukocytes may also indirectly affect tumor metastasis. Leukocytes attracted and activated by MCP-1 in the proximity of the endothelium can migrate across the endothelium and degrade extracellular matrix proteins, which separate the endothelium from the underlying layers of the vascular wall [9,10]. Such a process can markedly facilitate invasion of tumor cells, a process associated with the development of metastasis. To support the role of

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MCP-1 in tumor metastasis, it was demonstrated that levels of this chemokine were elevated in serum of ovarian cancer patients [6] and in urine of patients with bladder cancer [7]. In fact, the urinary MCP-1 levels were strongly correlated with tumor stage, grade, and distant metastasis [7].

Selected dietary fatty acids can modulate inflammatory responses in numerous tissues, including the vascular endothelium [11]. However, it appears that the effects mediated by individual fatty acids are very specific, and are influenced by diet and types of dietary fat. Among different dietary fatty acids, linoleic acid may play one of the most critical roles in induction of alterations of endothelial cell metabolism [11,12]. It was reported that this fatty acid can disrupt endothelial cell integrity, alter functions of gap-junctional proteins [13], increase levels of intracellular calcium, and induce cellular oxidative stress [14]. In clinical studies, a positive correlation was found between linoleic acid levels in the phospholipid fractions of human coronary arteries and ischemic heart disease [15] as well as between concentrations of linoleic acid in adipose tissue and the degree of coronary artery disease [16]. Evidence also indicates that dietary linoleic acid also can promote carcinogenesis. In fact, it was demonstrated that when the dietary content of linoleic acid exceeded 4–5% of total calories, any additional fat linearly increased chemically-induced tumor incidence [17,18]. In addition to its role in carcinogenesis, dietary linoleic acid can also enhance the metastatic formation of mammary tumors. For example, a linoleic acid-enriched diet increased the rate of metastasis of mammary cancer to the lung in rats [19]. However, detailed mechanisms of linoleic acid-stimulated cancer metastasis are not fully understood, and we hypothesize that induction of vascular endothelial cell inflammatory genes, such as genes encoding for adhesion molecules or chemokines, including MCP-1, may markedly contribute to carcinogenesis and cancer metastasis induced by this fatty acid.

Because of the importance of MCP-1 induction in vascular biology, and because of the involvement of linoleic acid in the pathology of the vascular endothelium, the present study was designed to examine the regulatory mechanisms of linoleic acid-induced MCP-1 gene expression in microvascular endothelial cells. We demonstrate that linoleic acid can trigger overexpression of the MCP-1 gene, leading to increased MCP-1 production, through an oxidative stress-related mechanism.

2. Methods

2.1 Endothelial cell culture

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky Medical Center). HMEC-1 were cultured in MCDB 131 media (Sigma, St. Louis, MO) enriched with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/ml hydrocortisone and 0.01 μg/ml epidermal growth factor in a 5% CO_2 atmosphere at 37°C. Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). The medium was enriched with linoleic acid as described previously [20].

In selected experiments, HMEC-1 were pretreated for 30 min with pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO) at the levels of up to 25 μM or with N-acetylcysteine (NAC, Sigma, St. Louis, MO) at the levels of up to 50 mM.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HMEC-1 were prepared according to the method of Beg *et al* [21] as described earlier [22]. Binding reactions were performed in a 20 μl volume containing 6 μg of nuclear protein extracts, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μg of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of ^{32}P-labeled specific oligonucleotides that contained the NF- κB sequence specific for the NF- κB site binding site in the MCP-1 promoter (5′-AGA GTG GGA ATT TCC ACT CA-3′). The resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using 0.25 × TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were employed in supershift experiments.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma, St. Louis, MO) and reverse-transcribed at 42°C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μl of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer [22]. For amplification of MCP-1 and of β-actin (a housekeeping gene), the following primer combinations were used: 5′-CAG CCA GAT GCA ATC AAT GC-3′ and 5′-GTG GTC CAT GGA ATC CTG AA-3′ (MCP-1; expecting 198-bp fragment; R&D Systems, Minneapolis, MN) and 5′-AGC ACA ATG AAG ATC AAG AT-3′ and 5′-TGT AAC GCA ACT AAG TCA TA-3′ (β-actin; expecting 188-bp fragment) [23]. The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated 20 times. Amplification was linear within the range of 15–25 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and

visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

2.4. Measurement of MCP-1 production

MCP-1 concentrations in cell culture supernatants were determined using a Quantikine® Human MCP-1 Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. This assay employs the quantitative sandwich enzyme immunoassay technique using a murine monoclonal antibody against human MCP-1 and a polyclonal secondary antibody conjugated with horseradish peroxidase. The minimum detectable concentration of MCP-1 was less than 5.0 pg/ml.

2.5. Statistical analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to compare responses among the treatments. The treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $p < 0.05$ was considered significant.

3. Results

3.1. Linoleic acid activates NF-κB binding in microvascular endothelial cells

NF-κB is an oxidative stress-responsive transcription factor, which is involved in transcriptional regulation of a variety of inflammatory genes [24]. In addition, activation of NF-κB can serve as a sensitive marker of oxidative stress and alterations in cellular redox status. To determine if linoleic acid can activate NF-κB in HMEC-1, cells were exposed up to 50 μM of this fatty acid for 2 h and NF-κB binding was analyzed by EMSA, using nuclear extracts from the treated cells. As shown in Figure 1, a slight endogenous activity of NF-κB was observed in control cultures (lane 2). However, when the HMEC-1 were stimulated with linoleic acid, a marked increase in NF-κB binding activity was detected (lanes 3–5). This binding was completely inhibited by an unlabeled competitor DNA containing the consensus NF-κB sequence (lane 6). In addition, the identity of NF-κB binding was confirmed by experiments in which nuclear extracts isolated from linoleic acid-treated cultures were incubated with antibodies against specific NF-κB subunits prior to adding the radioactive NF- κB oligonucleotide probe. As indicated, incubation with both anti-NF-κB p50 or anti-NF-κB p65 antibody resulted in a marked decrease in intensity of the NF-κB band (lanes 7 and 8).

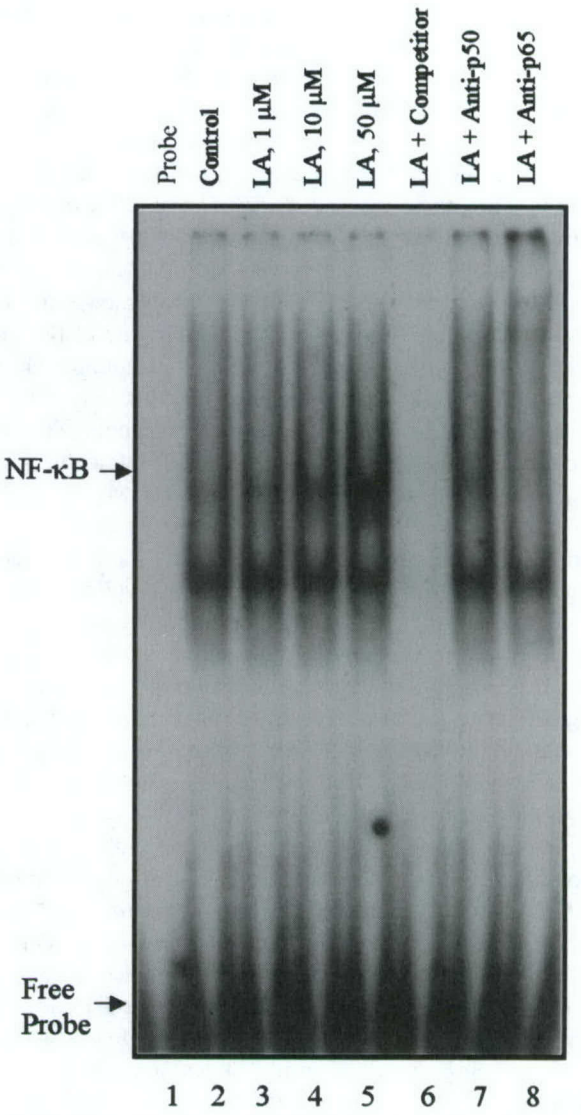

Fig. 1. Linoleic acid enhances NF-κB binding in human microvascular endothelial cells (HMEC-1). Cells were either untreated (lane 2) or treated with increasing doses of linoleic acid (lanes 3–5) for 2 h. Nuclear extracts were analyzed by EMSA. Competition study and supershift analysis were performed by the addition of excess unlabeled oligonucleotide (lane 6) and anti-NF-κB antibody (anti-p50 and anti-p65, lanes 7 and 8, respectively), using nuclear extracts from HMEC-1 stimulated by 50 μM of linoleic acid for 2 h.

3.2. Linoleic acid stimulates MCP-1 gene expression and protein production in microvascular endothelial cells

Figure 2 indicates the effects of linoleic acid on MCP-1 mRNA expression in HMEC-1 using a semi-quantitative RT-PCR technique. As indicated, low levels of MCP-1 mRNA were observed in control cell cultures. In addition, treatment of HMEC-1 with 50 μM of linoleic acid markedly and in a time-dependent way increased accumulation of MCP-1 mRNA (Figure 2A). Upregulation of the MCP-1 mRNA expression was already detected 1 h after linoleic acid treatment and reached the maximum levels at 3 and 4 h. Figure 2B indicates that

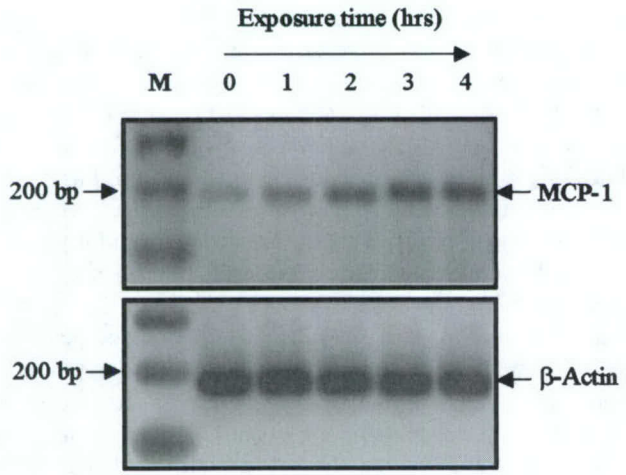

Fig. 2A. Time-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to 50 μM linoleic acid for up to 4 h. The levels of MCP-1 mRNA were determined by RT-PCR. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging. The predicted sizes of RT-PCR products for MCP-1 and β-actin (represented by arrows) are 198 bp and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder).

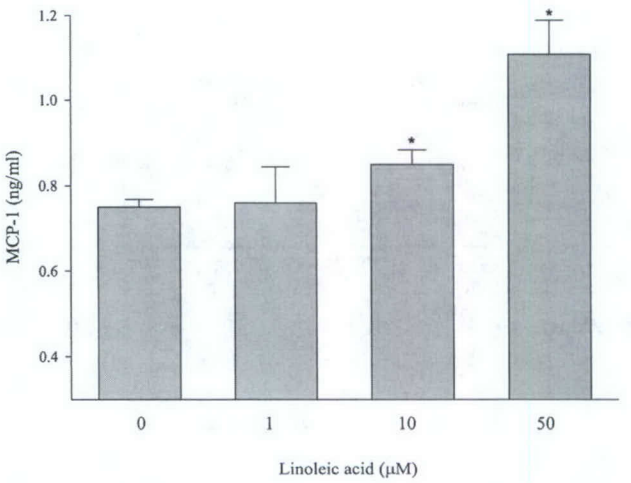

Fig. 3. Linoleic acid increases production of MCP-1 protein in human microvascular endothelial cells (HMEC-1). Cells were treated with increasing concentrations of linoleic acid for 16 h. Concentration of MCP-1 was measured by ELISA in the aliquots of culture media. Values represent mean ± SD. *Statistically significant compared to the control group ($P < 0.05$).

linoleic acid-induced stimulation of the MCP-1 mRNA is dose dependent. Maximal induction of the MCP-1 gene was detected in HMEC-1 exposed to linoleic acid at the dose of 50 μM.

The quantitative sandwich enzyme immunoassay technique was employed to determine whether linoleic acid-mediated induction of the MCP-1 gene is paralleled by a concomitant production of MCP-1 protein. Concentration of MCP-1 protein was determined in culture supernatants from HMEC-1 treated with different doses of linoleic acid for 16 h (Figure 3). Consistent with the data on MCP-1 gene expression, treatment with linoleic acid resulted in a dose-dependent upregulation of MCP-1 protein levels. Significant elevations of MCP-1 levels were observed in cultures exposed to 10 and 50 μM of linoleic acid.

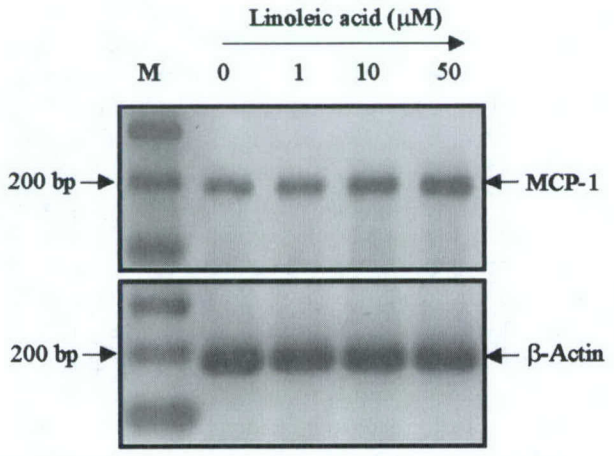

Figure 2B. Dose-dependent upregulation of MCP-1 mRNA expression by linoleic acid in human microvascular endothelial cells (HMEC-1). Cells were exposed to increasing concentrations of linoleic acid for 4 h. The levels of MCP-1 mRNA were determined as described in the legend to Figure 2A.

3.3. Antioxidants attenuate linoleic acid-induced MCP-1 gene expression

To determine whether linoleic acid-mediated MCP-1 gene expression is mediated by an oxidative stress-related mechanism, HMEC-1 were pretreated for 30 min either with pyrrolidine dithiocarbamate (PDTC) or with N-acetylcysteine (NAC), followed by a 4 h treatment with 50 μM of linoleic acid. Both PDTC and NAC are widely used as antioxidant compounds to study redox regulation of intracellular signaling pathways and of cell function [25,26]. As shown in Figure 4A, PDTC attenuated linoleic acid-induced MCP-1 mRNA levels. Similar effects were observed when HMEC-1 were pretreated with NAC prior to exposure to linoleic acid (Figure 4B).

4. Discussion

Linoleic acid is the major dietary fatty acid present in high concentrations in corn, soy, sunflower, or safflower oils. It is estimated that it provides approximately 7–8% of the average dietary energy intake [27]. Such a high consumption of linoleic acid may markedly affect endothelial cell metabolism. It is widely recognized that the lipid composition of plasma lipoproteins is closely related to dietary fat intake [28]. In addition, it has been proposed that hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism

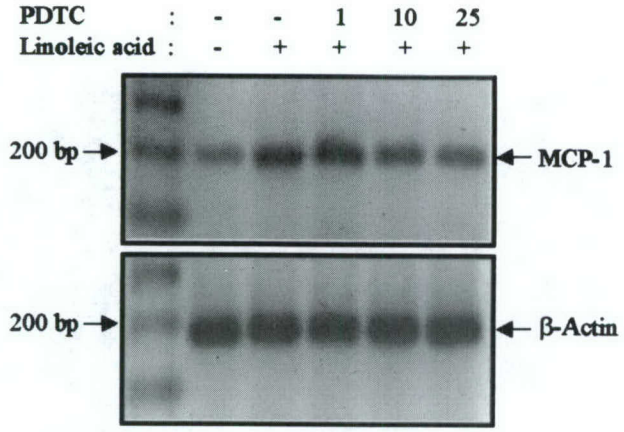

Fig. 4A. Pyrrolidine dithiocarbamate (PDTC) inhibits linoleic acid-induced induction of MCP-1 mRNA in human microvascular endothelial cells (HMEC-1). Cells were pretreated with indicated amounts of PDTC for 30 min before a 4 h treatment with 50 μM linoleic acid and analyzed for MCP-1 mRNA by RT-PCR as described in the legend to Figure 2A.

that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions in the proximity to the endothelium [29,30]. Therefore, the fatty acids which are exposed to endothelial cells are correlated with the type of fat that is being consumed [28,31].

Linoleic acid may be one of the most important dietary factors which can activate the vascular endothelium, a process which is involved in a variety of pathological conditions, such as early atherosclerotic changes or induction of cancer metastasis. Research from our laboratories demonstrated that exposure of endothelial cells to this fatty acid can induce profound inflammatory responses, demonstrated by increased production of adhesion molecules and inflammatory cytokines [32–34]. In line with these earlier reports, the results of the present study indicate that exposure of

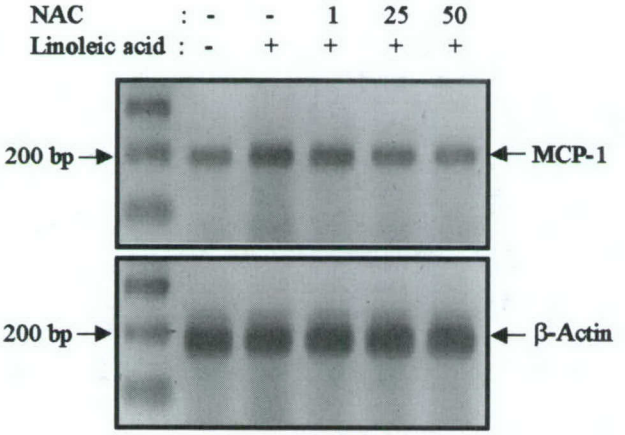

Figure 4B. N-Acetylcysteine (NAC) inhibits linoleic acid-induced induction of MCP-1 mRNA in human microvascular endothelial cells (HMEC-1). Cells were pretreated with indicated amounts of NAC for 30 min before a 4 h treatment with 50 μM linoleic acid and analyzed for MCP-1 mRNA by RT-PCR as described in the legend to Figure 2A.

HMEC-1 to linoleic acid can induce MCP-1 gene expression through an oxidative stress-related mechanism. We demonstrated that pretreatments of endothelial cells either with PDTC or with NAC attenuated linoleic acid-induced elevation of the MCP-1 mRNA levels (Figure 4). Several lines of evidence can explain this phenomenon. For example, polyunsaturated fatty acids, and in particular linoleic acid, are potent prooxidants. In fact, linoleic acid is considered to be the predominant substrate for lipid peroxidation processes both in lipoproteins, such as low-density lipoproteins (LDL), as well as in tissues [35]. Linoleic acid was demonstrated to a) enhance radical adduct formation in endothelial cells exposed to iron-induced oxidative stress [36], b) decrease glutathione levels [20], and c) increase peroxisomal β-peroxidation [37], a pathway that leads to the production of hydrogen peroxide. Degradation of linoleic acid *via* the cytochrome-P450 pathway also can lead to formation of highly prooxidative and proinflammatory derivatives, such as epoxides and diol metabolites [38]. In support of the hypothesis that expression of human MCP-1 might be regulated by oxidative stress-related mechanisms it was demonstrated that red wine with high antioxidant capacity can inhibit MCP-1 expression and reduce neointimal thickening after balloon injury of the aorta in cholesterol-fed rabbits [39].

Evidence indicates that not only linoleic acid but also a variety of its oxidative derivatives can induce profound proinflammatory responses [38,40]. However, the present study indicated that already a 2 h exposure to linoleic acid was sufficient to markedly elevate the MCP-1 mRNA levels in HMEC-1. Such a very short exposure time suggests that induction of the MCP-1 gene may be caused by a direct effect of linoleic acid rather than by its oxidative metabolites.

Transcriptional mechanisms of linoleic acid-induced MCP-1 gene expression are not fully understood; however, they may involve activation of transcription factors whose binding sites are present in the promoter region of the MCP-1 gene. Evidence indicates that putative binding sites for NF-κB, AP-1, SP-1 and GAS exist in the 5′-flanking region of the human MCP-1 gene [41]. Although activation of these transcription factors appears to be redox-responsive, they are regulated by different and specific mechanisms. It is generally accepted that activation of NF-κB is regulated by increased cellular oxidative stress and/or alterations of glutathione metabolism [42,43]. Evidence indicates that exposure of endothelial cells to linoleic acid can markedly affect glutathione levels. In fact, we observed a significant decrease in cellular glutathione content and increased ratio between oxidized and reduced glutathione in peripheral endothelial cells exposed to this fatty acid [20]. In addition to linoleic acid-induced activation of NF-κB, we have evidence that treatment with this fatty acid can markedly stimulate NF-κB-dependent transcription [14,44]. However, our earlier reports on vascular effects of linoleic acid have been based on cells isolated from major vascular vessels, such as pulmonary artery [14,20,33,38,44] or um-

bilical veins [22,32,34]. It is well known that endothelial cells from different tissues and vessels can differ markedly in their structure and functions [45]. In the present study, we report that linoleic acid can activate NF-κB in microvascular endothelial cells, i.e., the type of endothelial cells that provide a most relevant experimental model to study vascular mechanisms of cancer metastasis. In addition, it should be noted that in the present study linoleic acid-mediated NF-κB activation was detected using the NF- κB oligonucleotide probe specific for the NF-κB binding site of the human MCP-1 promoter region.

AP-1 is another transcription factor that is activated by alterations of cellular redox status. However, the specific mechanisms of such activation appear to be complex. AP-1 is composed of the Jun and Fos gene products, which can form heterodimers (Jun/Fos) or homodimers (Jun/Jun). It has been demonstrated that under specific experimental conditions, both oxidants and antioxidants can lead to activation of this transcription factor [46,47]. For example, oxidation of cysteine residues of c-Fos and c-Jun (Fos Cys-154 and Jun Cys-272, respectively) can convert the AP-1 subunits into inactive forms and inhibit binding activity of this transcription factor [48]. However, oxidative stress also can induce the mitogen-activated protein kinase (MAPK) cascade which can lead to AP-1 activation [49]. Linoleic acid and its oxidative derivatives can stimulate both c-Fos and c-Jun mRNA expression, as well as activate MAPK in rat aortic smooth muscle cells [50]. In addition, in support of the possible involvement of NF-κB and AP-1 activation in linoleic acid-induced MCP-1 gene in HMEC-1, the critical role of these transcription factors in MCP-1 gene expression was demonstrated in cells stimulated with tumor necrosis factor-α (TNF-α) [51,52].

The promoter region of the MCP-1 gene also contains GAS and SP-1 binding sites [41,53]. However, their possible involvement in linoleic acid-induced overexpression of the MCP-1 gene is not fully understood. It is known that the transcription factor STAT1α specifically interacts with GAS binding sites. Our unpublished observations indicate that activation of STAT1α can be regulated by cellular oxidative status. However, there is no existing evidence whether this transcription can be activated by linoleic acid treatment in cultured microvascular endothelial cells. In addition, evidence indicates that activation of the SP-1 transcription factor can be regulated by the cellular redox status and that it plays a critical role in interleukin-4-mediated induction of the vascular adhesion molecule-1 (VCAM-1) gene expression [54]. However, at the present time it is unknown if a similar mechanism also is involved in linoleic acid-mediated overexpression of the MCP-1 gene in HMEC-1.

In conclusion, the present study provides compelling evidence that linoleic acid can induce MCP-1 expression in human microvascular endothelial cells, a cell model used for studying mechanisms of cancer metastasis. These data may contribute to a better understanding how dietary lipids can induce production of the inflammatory mediators in the microvasculature and contribute to a variety of pathological alterations, such as cancer metastasis.

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Liposome-Mediated High-Efficiency Transfection of Human Endothelial Cells

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Gene transfer · Cell culture · Vasculature

Abstract
Liposome-mediated transfection of endothelial cells provides a valuable experimental technique to study cellular gene expression and may also be adapted for gene therapy studies. However, the widely recognized disadvantage of liposome-mediated transfection is low efficiency. Therefore, studies were performed to optimize transfection techniques in human endothelial cells. The majority of the experiments were performed with primary cultures of human umbilical vein endothelial cells (HUVEC). In addition, selected experiments were performed using human brain microvascular endothelial cells and human dermal microvascular endothelial cells. To study transfection rates, HUVEC were transfected with the pGL3 vector, containing the luciferase reporter gene, complexed with several currently available liposomes, such as different Perfect Lipid (pFx) mixtures, DMRIE-C, or lipofectin. The optimal transfection rate was achieved in HUVEC transfected for 1.5 h with 5 μg/ml of DNA plasmid in the presence of 36 μg/ml of pFx-7. In addition, transfection with the VR-3301 vector encoding for human placental alkaline phosphatase revealed that, under the described conditions, transfection efficiency in HUVEC was approximately 32%. Transfections mediated by other liposomes were less efficient. The usefulness of the optimized transfection technique was confirmed in HUVEC transfected with NF-κB or AP-1-responsive constructs and stimulated with TNF or LPS. We conclude that among several currently available liposomes, pFx-7 appears to be the most suitable for transfections of cultured human endothelial cells.

Introduction

Cellular transfections (physical-chemical methods of introducing genes into cells) provide powerful experimental tools to study gene regulation in vivo and in vitro [1]. In addition, transfection techniques are used to deliver foreign DNA in gene therapy strategies [2, 3]. Stable transfections refer to the production of a population of cells in which the gene of interest is stably expressed in the cell. Thus, the gene is not only introduced into the cell but also is integrated into the host DNA and reproduced during cell cycles or cell division. The second general type of transfection is transient transfection, during which plasmid DNA is introduced into a cell population but no stable cell lines are isolated. Instead, gene expression is studied shortly after the transfection procedure, usually within 24–72 h [4]. The advantage of the second approach is the simplicity of the technique and the fact that the same

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preparation of DNA can be introduced into various cell types. Because cellular membranes create barriers for large and highly charged DNA molecules to enter cellular compartments, several techniques have been developed to facilitate cellular transfections. Transfection methods include calcium-phosphate precipitation, electroporation, detergent-DNA complexes, DNA-DEAE complexes, microinjection, virus-mediated transfection, introduction of DNA via particle bombardment and lipid-mediated transfection [2, 5]. In transfections performed in vitro in cultured cells, cationic lipids have become standard carriers of plasmid DNA [6].

Endothelial cells are a promising target in somatic gene therapy in cardiovascular disorders, ischemic disease [7] and cancer [8, 9], since the endothelium is involved in these pathological stages and endothelial cells are accessible for gene transfer via circulation [10]. Several experimental and clinical studies have demonstrated the therapeutic potential of somatic gene therapy in vascular diseases. For example, in the treatment of restenosis, positive results were obtained when animals were transfected with the genes encoding for vascular endothelial growth factor, nitric oxide synthase, thymidine kinase, retinoblastoma, growth arrest or antisense oligonucleotides against transcription factors [10, 11]. In atherosclerosis, gene therapy strategies have been used in the treatment of vascular proliferation, endothelial dysfunction, thrombosis, and ischemia as well as in modification of the blood/biomaterial interface [12]. It has also been reported that transfer of genes encoding for cyclooxygenase and endothelial nitric oxide synthase can protect against intimal hyperplasia in angioplasty-injured carotid arteries [13]. Clinical trials indicated that substantial therapeutic benefits could be obtained by intramuscular injections of naked DNA plasmid encoding for human vascular endothelial growth factor in patients with severe peripheral arterial disease [14].

The most efficient transgene expression can be achieved by using adenoviruses [15]. In fact, with adenovirus vector, recombinant genes can be delivered to approximately 100% of endothelial cells of normal human vessels in organ cultures [16]. However, adenoviral vectors can induce injury to the vessel wall. For example, in arteries transduced with replication-defective adenoviral vector AdRSVn-LacZ, a marked accumulation of macrophages and increased intimal cellularity were reported. In addition, in hypercholesterolemic cynomolgus monkeys, this vector caused an increase in vessel wall inflammation and progression of early atherosclerotic lesions [17]. Viral transduction can also induce changes in endothelial cell phenotype [18]. Therefore, nonviral transfections, including cationic liposomes, remain attractive carriers to facilitate the entry of foreign DNA into endothelial cells.

The aim of the present study was to optimize a transfection technique using different, currently available cationic liposomes in cultured human endothelial cells. Transfection rate was established using liposomes complexed with the pGL3 vector, driven by the simian virus 40 (SV40) promoter and containing the luciferase reporter gene. In addition, the efficiency of transfection was studied by employing the VR-3301 vector driven by the cytomegalovirus (CMV) promoter ligated to the human placental alkaline phosphatase reporter gene (hpAP). We found that cultured human endothelial cells can be efficiently transfected.

Materials and Methods

Endothelial Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously [19]. They were maintained in growth medium containing M199, 25 m*M* HEPES, 54.3 U/ml heparin, 2 m*M* *L*-glutamine, 1 μ*M* sodium pyruvate, 200 U/ml penicillin, 200 μg/ml streptomycin, 0.25 μg/ml amphotericin B (all reagents from Gibco BRL, Grand Island, N.Y., USA), 40 μg/ml endothelial cell growth supplement (ECGS, Becton Dickinson, Bedford, Mass., USA), and 20% FBS (HyClone Laboratories, Inc., Logan, Utah, USA).

Cells were determined to be endothelial by their cobblestone morphology and uptake of fluorescent labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc., Eugene, Oreg., USA). All experiments were conducted with cells from passage two.

Selected experiments also were performed using human aortic endothelial cells (HAEC), immortalized human brain microvascular endothelial cells (HBMEC) and immortalized human dermal microvascular endothelial cells (HMEC-1). HAEC were purchased from Clonetics Corp., (Walkersville, Md., USA) and cultured in medium supplied by the manufacturer. HBMEC (a generous gift from Dr. M. Fiala, UCLA School of Medicine) were isolated from a brain biopsy of an adult female with epilepsy and immortalized by transfection with SV40 large-T antigen. They were cultured in RPMI-1640 medium (Gibco BRL), supplemented with 10% FBS (HyClone Laboratories), 10% NuSerum IV (Becton Dickinson), 1% nonessential amino acids, 1% vitamins, 5 U/ml heparin, 1 m*M* sodium pyruvate, 2 m*M* *L*-glutamine (all reagents from Gibco BRL), and 30 μg/ml ECGS (Becton Dickinson) [20, 21].

HMEC-1 (a generous gift from Dr. E. Smart, University of Kentucky) were isolated from dermal microvessels and immortalized by transfection with SV40 large-T antigen. They were cultured in MCDB-131 medium (Gibco BRL) supplemented with 10% FBS (HyClone Laboratories), 200 U/ml penicillin, 200 μg/ml streptomycin (Gibco BRL), 10 ng/ml endothelial growth factor (Calbiochem, San Diego, Calif., USA) and 1 mg/ml hydrocortisone (Sigma).

Each experiment was performed at least in triplicate on at least four (and up to 12) independent cultures.

Liposome Carriers for Transient Transfection and Transfection Procedure

The PerFect Lipid Transfection kit (Invitrogen, Carlsbad, Calif., USA), DMRIE-C, and lipofectin (Gibco BRL, Grand Island, N.Y., USA) were used for transfections of endothelial cells. The PerFect Lipid Transfection kit provides eight different compositions of lipids (pFx 1–8), and each of these lipids was employed in the present study. Molecular weights of different pFx mixtures vary from 847 (pFx-4) to 2,617 (pFx-8). The molecular weight of pFx-7, the liposome used in the majority of our experiments, is 1,011. DMRIE-C (molecular weight 646) was used because it resembles a lipid carrier which was previously used successfully for endothelial cell transfections [5]. Lipofectin (molecular weight 669.5), which was employed in our earlier study [22], is widely used in transfection of endothelial cells. Selected experiments also were performed using cytofectin GCV (Glen Research, Sterling, Va., USA), DAC-30 (Eurogentec, Sersing, Belgium), and SuperFect (Qiagen, Valencia, Calif., USA). These additional liposomes were selected based on a recent report which demonstrated that cytofectin GCV or SuperFect can mediate uptake of antisense oligonucleotides in cultured human iliac artery endothelial cells with high efficiency [23].

For transfection studies, endothelial cells were seeded in 12-well plates and grown to 50–60% confluency in normal growth medium. To perform transfections, aliquots of normal M199 were mixed with different concentrations of specific lipid carriers in polystyrene tubes, mixed with plasmid DNA and incubated at 37°C for 30 min to allow the formation of DNA-lipid complexes. Endothelial cell cultures were washed three times with M199 to remove serum, and 1 ml of transfection solution was added to each well of the 12 well plates. Controls consisted of endothelial cells incubated with plasmid DNA alone or liposomes complexed with a carrier plasmid. After incubation, transfection solutions were aspirated and replaced with growth medium. Cells were maintained in these conditions for 48 h before assays for reporter genes were performed.

pGL3 Vector and Luciferase Reporter Gene Assay

To monitor the transfection rate, endothelial cells were transfected with individual liposomes complexed with the pGL3 Luciferase Reporter Vector (Promega, Madison, Wisc., USA). This vector contains the SV40 promoter and enhancer sequence and firefly luciferase as a reporter gene. Following the transfection process, luciferase activity was measured by Luciferase Assay System (Promega) according to the instructions supplied by the manufacturer. Briefly, culture media were removed and cells were washed three times with PBS and incubated for 10 min with 60 µl of Cell Culture Lysis reagent. Attached cells were then scraped, centrifuged to remove membrane debris, transferred to new tubes, and stored at –80°C until analysis. For luciferase assay, 10 µl of the cell extracts were mixed with 100 µl of Luciferase Assay Reagent containing luciferin and ATP in a luminometer with automatic injection. Light emission was measured every 0.5 s, for 10 s. Values are expressed in RLU/µg protein. Cellular proteins were measured using Bradford reagent (Bio-Rad, Hercules, Calif., USA).

VR-3301 Vector and Alkaline Phosphatase Reporter Gene Assay

To establish transfection efficiency, endothelial cells were transfected with VR-3301 vector (Vical Inc., San Diego Calif., USA) mixed with pFx-7, DMRIE-C or lipofectin. The VR-3301 vector contains CMV promoter/enhancer which regulates expression of the hpAP gene. Transfected endothelial cells were fixed in 4% paraformaldehyde for 1 h and then washed 3 times with PBS. Following heat inactivation of endogenous alkaline phosphate isoenzymes of nonplacental origin (30 min at 65°C), cells were stained for hpAP using an azo dye coupling technique [24]. Briefly, 0.2 ml of naphtol AS-MX phosphate (0.25% alkaline solution, Sigma) were mixed with 4.8 ml of 0.1 *M* Tris-HCl buffer (pH 10.0) and 10 mg of fast red TR salt (Sigma). The stain mixture was filtered immediately before use, and cells were stained for the presence of hpAP for 15 min at room temperature. In independent sets of experiments, fluorescence of transfected cells was determined either by flow cytometry (in cell suspension) or fluorescent microscopy (in cells cultured on glass-bottom dishes) using rhodamine filter sets. Data are expressed as a percentage of cells in which activity of hpAP was detected.

Employment of the Optimized Transfection Conditions to Study Activation of Transcription Factors in Endothelial Cells

To determine whether the optimized transient transfection technique is useful in studies on transcription factor activation in endothelial cells, HUVEC were transfected for 1.5 h with 5 µg of NF-κB or AP-1 reporter plasmids (Stratagene, La Jolla, Calif., USA) mixed with 36 µg/ml of pFx-7. NF-κB responsive plasmid contained five repeats of NF-κB enhancer elements, and AP-1-responsive plasmid contained seven repeats of AP-1 enhancer elements, linked to basic TATA element and the firefly luciferase reporter gene. Following transfection, cells were incubated in normal growth medium for 24 h. Then, HUVEC were treated with either TNF (10 ng/ml) or LPS (1 µg/ml) in a medium containing 10% FBS for 24 h. At the end of the incubation time, cells were washed with PBS, lysed, and measured for luciferase activity using Luciferase Assay Reagent (Promega).

Cell Proliferation (5-Bromo-2′-Deoxyuridine Incorporation Assay)

Endothelial cell proliferation was determined by the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay according to the procedure supplied by the manufacturer (Roche Diagnostics, Mannheim, Germany). This assay takes advantage of the incorporation of BrdU, instead of thymidine, into the DNA of proliferating cells. Briefly, immediately following transfections, endothelial cells were incubated for 12 h with 10 µ*M* BrdU diluted in normal growth medium. Then, cultures were fixed and incubated with monoclonal anti-BrdU antibody labeled with peroxidase. Following a 30-min incubation, tetramethylbenzidine was added as a substrate for peroxidase and, after a 10-min interval time required for color development, absorbance was read at 370 nm. The results were expressed as percentage of control.

Statistical Analysis

Statistical analysis was performed using SYSTAT 8.0 (SPPS Inc., Chicago, Ill., USA). One-way or two-way ANOVA was used to compare the mean values among the treatments. Two-way ANOVA was employed in statistical analysis of all experiments which included at least two variables, such as time and different treatment factors. When the overall F values were significant, ANOVA was followed by a posthoc Bonferroni test to compare means from different treatments. Statistical probability of $p < 0.05$ was considered significant.

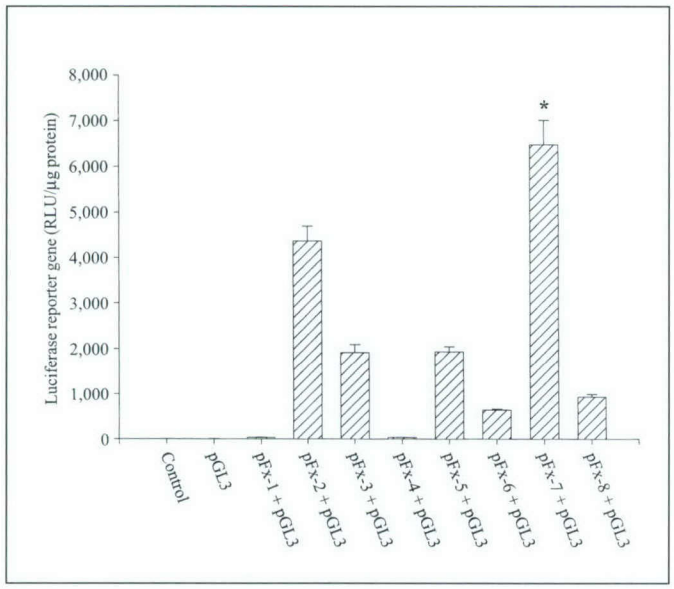


Fig. 1. Comparison of transfection rates mediated by different pFx mixtures. Cells were transfected for 3 h with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of individual pFx lipids. Transfections were followed by a 48-hour recovery period in normal growth medium, after which the reporter gene assay was performed. Values are mean ± SEM. *Values in cultures transfected by pFx-7 are significantly higher than values from groups transfected with other pFx lipids.

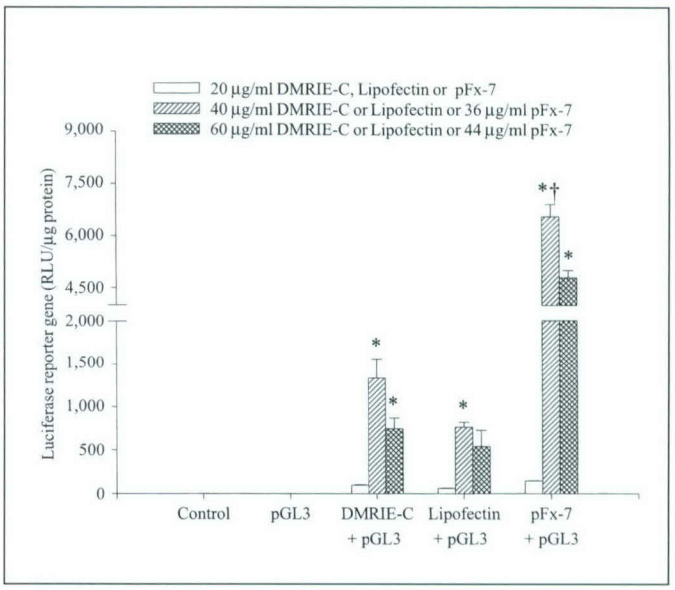


Fig. 2. The effect of liposome concentrations on transfection rates in HUVEC. Cells were transfected for 1.5 h with 5 μg/ml of the pGL3 vector complexed with different concentrations of pFx-7, DMRIE-C or lipofectin. Values are mean ± SEM. *Values are statistically significant as compared to the values in the group transfected with the preceding concentration of a given liposome. †Values in cultures transfected in the presence of 36 μg/ml pFx-7 are significantly higher than transfection rates in other experimental groups.

Results

Transient Transfection Rates Mediated by Different pFx Liposomes

To determine the most effective pFx liposome as a mediator of transient transfection of endothelial cells, HUVEC were transfected with 5 μg/ml of the pGL3 vector complexed with 36 μg/ml of each liposome provided in the PerFect Lipid Transfection kit. Figure 1 indicates transfection rates, as determined by luciferase activity, mediated by individual pFx liposomes. Transfections were performed for 3 h, followed by a 48-hour recovery process. Only minimal transfection rates (range of 3–7 RLU/μg protein) were determined in HUVEC exposed to the pGL3 vector alone. Except for pFx-1 and pFx-4, all remaining pFx liposomes successfully mediated transfection of HUVEC. However, the most marked transfection rate was observed in cells transfected with pFx-7. Therefore, optimization of transfection conditions was performed with this liposome.

Comparison of Transfection Rates Mediated by DMRIE-C, Lipofectin, or pFx-7 and Optimization of Liposome Concentrations

DMRIE-C reagent and lipofectin are commercially available liposomes, widely used to initiate transient or stable transfections. To establish the most suitable liposome carrier and the optimal liposome concentration for transient transfection of endothelial cells, the pGL3 vector (5 μg/ml) was complexed with different concentrations of DMRIE-C, lipofectin or pFx-7. Transfections were performed for 1.5 h, followed by a 48-hour recovery period. Results of these experiments are reflected in figure 2. Liposomes at the concentrations of 20 μg/ml (or lower – data not shown) appeared to be ineffective in HUVEC transfection. However, an increase in liposome concentrations from 20 to 40 μg/ml for DMRIE-C or lipofectin and to 36 μg/ml for pFx-7 resulted in an increase of transfection rates, as measured by luciferase activity. In particular, a dramatic increase (approximately 250 times) in transfection rate was detected in HUVEC transfected with pGL3 complexed with pFx-7 at the concentration of 36 μg/ml. The rate of transfection mediated by this con-

centration of pFx-7 was approximately 8.5 times higher compared to transfection induced by 40 μg/ml lipofectin. In addition, the transfection rate achieved by pFx-7 exceeded that mediated by 40 μg/ml DMRIE-C by almost 5 times. Further increase in concentrations of DMRIE-C, lipofectin or pFx-7 decreased transfection rates. It appears that a marked cytotoxicity observed in endothelial cell cultures exposed to high doses of liposomes was responsible for this phenomenon.

In separate experiments, transfection rates mediated by pFx-7 at the dose of 36 μg/ml were compared to those mediated by cytofectin GCV (used at the concentration range of 1–40 μg/ml), DAC-30 (concentration range of 5–30 μg/ml), and SuperFect (concentration range of 20–80 μg/ml). Among these liposomes, transfection of HUVEC mediated by pFx-7 also resulted in the highest transfection rates (data not shown).

Liposome-Mediated Toxicity in Cultured Endothelial Cells

When introduced into cell cultures, liposomes can induce cytotoxic effects which depend on lipid concentration and transfection time. Therefore, their toxic effects were also measured in cultured endothelial cells. BrdU incorporation assay, which reflects cell proliferation, was used in these studies. As indicated in table 1, treatments with lipofectin appeared to be most toxic in cultured endothelial cells. Diminished incorporation of BrdU was observed in endothelial cells incubated with 40 or 60 μg/ml of lipofectin for as short as 1 h. In addition, when cells were treated with lipofectin for 3 h, even lower doses of this liposome decreased proliferation of endothelial cells.

Transfection mediated by pFx-7 resulted in a moderate toxicity. Endothelial cell proliferation was not statistically decreased when this liposome was used at the doses of up to 36 μg/ml for 1 or 1.5 h. However, a higher dose (i.e., 44 μg/ml) of pFx-7 as well as a 3 h incubation time markedly diminished incorporation of BrdU in transfected HUVEC (table 1). In general, the most marked cytotoxicity was observed when endothelial cells were exposed to high doses of liposomes for 3 h. Liposome-mediated toxic effects similar to those detected in HUVEC were observed in cultures of HAEC (data not shown).

Although 1.5-hour treatments with liposomes at concentrations which mediated the optimal transfection rates as reported in figure 2 did not affect BrdU incorporation, they resulted in morphological changes of cultured endothelial cells. Because the character of these changes was similar for all studied liposomes, they are documented only for pFx-7, the liposome which produced the highest transfection rates in HUVEC. Figure 3A reflects morphological alterations of HUVEC, as observed under a phase-contrast microscope, after a 1.5-hour incubation with 36 μg/ml pFx-7 complexed with 5 μg/ml of the pGL3 vector. Cytotoxic effects of this complex included cellular shrinkage and detachment. However, a 48-hour recovery period following transfection, during which cells were maintained in normal growth medium, allowed HUVEC to regain normal morphological features. This phenomenon is shown in figure 3B, a photograph of the same culture as depicted in figure 3A, but taken after the recovery period.

Table 1. Toxic effects of different transfection carriers as measured by the incorporation of BrdU assay

Transfection carrier	Exposure time		
	1 h	1.5 h	3 h
Lipofectin, μg/ml			
10	98.7 ± 3.94	94.1 ± 1.97	69.9 ± 0.82[a, b]
20	97.1 ± 4.70	91.7 ± 2.57	59.4 ± 4.75[a, b]
40	79.4 ± 8.49[a]	74.1 ± 4.96[a, c]	47.3 ± 4.69[a, b]
60	60.9 ± 4.06[a]	54.3 ± 0.33[a, c]	47.4 ± 5.47[a]
DMRIE-C, μg/ml			
10	98.5 ± 6.37	90.5 ± 1.31	96.6 ± 2.10
20	105.2 ± 4.45	87.3 ± 7.32	76.2 ± 1.47[a, c]
40	101.7 ± 2.69	86.1 ± 1.38[a]	74.4 ± 3.17[a, b]
60	102.4 ± 1.22	81.2 ± 2.01[a, b]	64.7 ± 2.55[a, b]
pFx-7, μg/ml			
12	98.4 ± 3.30	92.2 ± 3.07	93.5 ± 4.28
24	95.1 ± 2.63	86.9 ± 2.30	85.6 ± 3.22
36	88.7 ± 5.82	84.2 ± 4.79	66.7 ± 0.98[a, c]
44	65.0 ± 3.11[a, c]	61.5 ± 4.94[a–c]	52.6 ± 2.69[a, c]

Values are mean ± SEM and are expressed as percentage of control.

[a] Statistically different as compared to control, i.e., non-transfected cells.

[b] Statistically different as compared to the values in the group transfected with the same concentration of a given liposome for the preceding exposure time.

[c] Statistically different as compared to the values in the group transfected for the same exposure time with the preceding concentration of a given liposome.

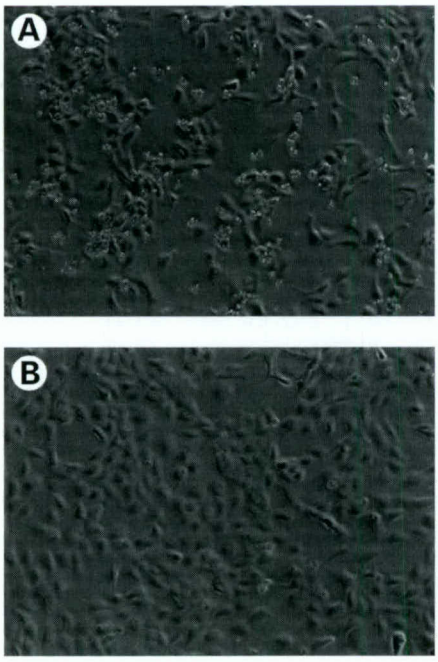


Fig. 3. The effect of pFx-7-mediated transfection on HUVEC morphology as observed under a phase-contrast microscope. Cells were transfected for 1.5 h with the pGL3 vector complexed with 36 μg/ml of pFx-7. **A** Cell morphology at the end of the 1.5-hour transfection period. **B** Cell morphology at the end of the 48 hour recovery period in which cells were maintained in normal medium.

Optimization of Plasmid DNA Concentration and Transfection Time for Transient Transfection of Endothelial Cells

Both the amount of plasmid DNA used for transfection and transfection time are important factors which can determine the transfection rate. Figure 4 shows transfection rates in HUVEC transfected with different amounts of the pGL3 vector complexed with pFx-7 at the concentration of 36 μg/ml as well as with DMRIE-C or lipofectin at the concentration of 40 μg/ml. Maximum transfection rate was observed in cells transfected with 5 μg/ml of plasmid DNA. In cells transfected with either 2 or 10 μg pGL3/ml, transfection rates were minimal as compared to 5 μg/ml of the pGL3 vector.

To determine the optimal transfection time, HUVEC were transfected for 1, 1.5 or 3 h, followed by a 48-hour recovery period. Time-dependent effects on liposome-mediated transfection are also shown in figure 4. As reflected in this figure, most successful HUVEC transfections resulted from 1.5-hour transfection time. Transfection rates in cells exposed to liposomes for that period of time were constantly higher than those in HUVEC transfected for 1 h. In addition, extension of transfection time to 3 h did not result in higher transfection rates. It appears that cytotoxicity of liposomes, as reported in table 1, could affect transfection rates in HUVEC transfected for 3 h.

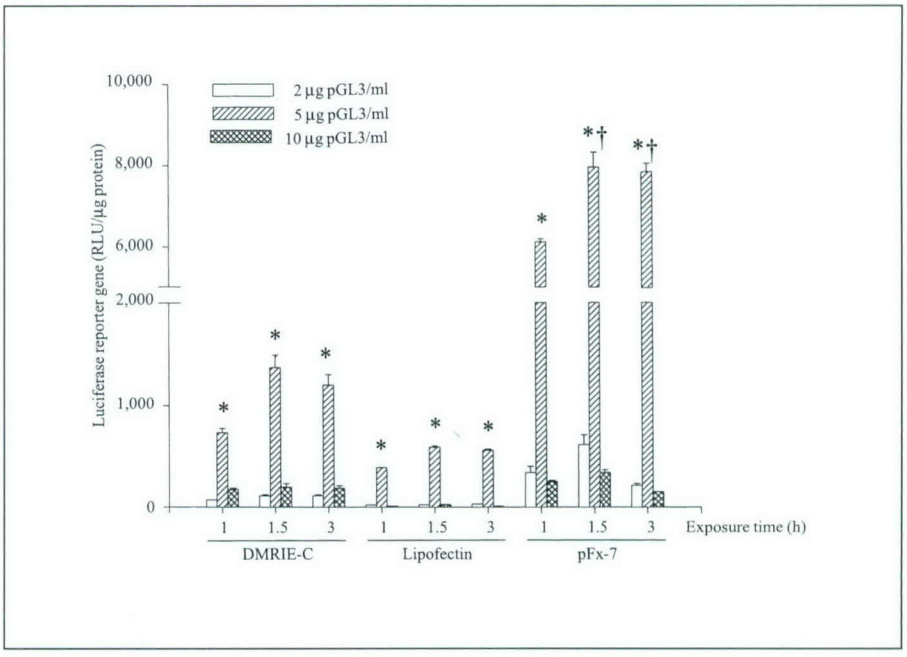
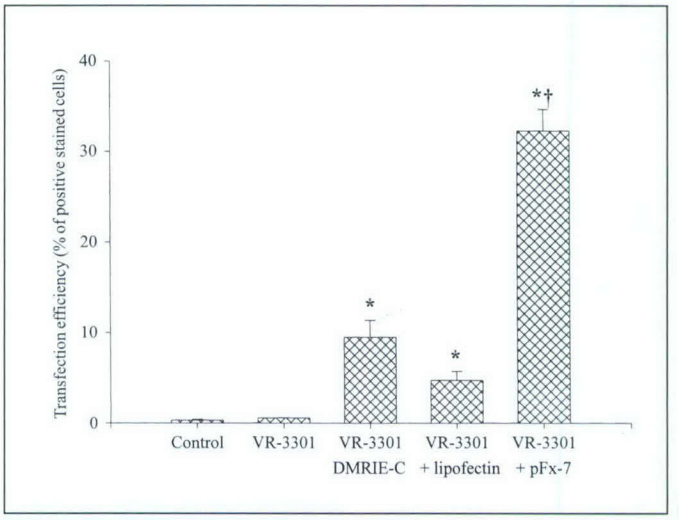


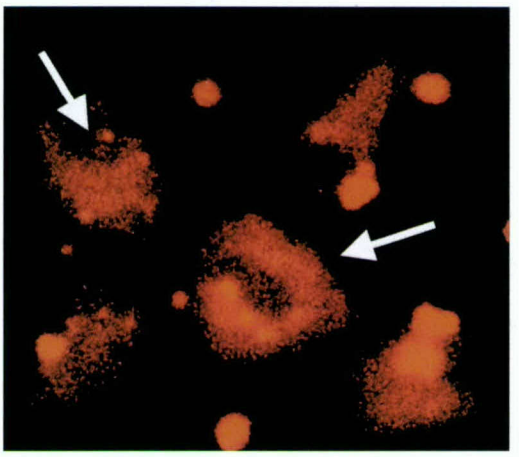
Fig. 4. Comparison of transfection rates mediated by DMRIE-C, lipofectin, or pFx-7 under different concentrations of plasmid DNA and transfection times. HUVEC were transfected for 1, 1.5 or 3 h with different concentrations of the pGL3 vector complexed with DMRIE-C or lipofectin at the concentration of 40 μg/ml or with pFx-7 at the concentration of 36 μg/ml. Values are mean ± SEM. Luciferase activities in control (nontransfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. *Values in cultures transfected with 5 μg pGL3/ml are significantly higher than values from groups transfected with other amounts of plasmid DNA. †Values in cultures transfected for 1.5 or 3 h in the presence of 36 μg pFx-7/ml complexed with 5 μg pGL3/ml are significantly higher than transfection rates in other experimental groups.

Efficiency of Transient Transfection in Endothelial Cells

Previously described experiments allowed us to determine the optimal transfection conditions for HUVEC using individual liposomes , i.e., pFx-7 at the concentration of 36 μg/ml, DMRIE-C or lipofectin at the concentration of 40 μg/ml, a transfection time of 1.5 h, and plasmid DNA concentration of 5 μg/ml. Using these experimental settings, transfection efficiency was measured by determination of activity of human placental alkaline phosphatase (hpAP) in HUVEC transfected with the VR-3301 vector, encoding for hpAP, and complexed with pFx-7, DMRIE-C or lipofectin. A fluorescent marker of hpAP activity, the fast red TR salt, was employed in these studies, and fluorescence was measured by either flow cytometry (in cell suspension) or fluorescent microscopy. Figure 5A shows the results of the quantitative analysis of transfection efficiency performed by flow cytometry. Under the described conditions, transfection efficiency in HUVEC mediated by pFx-7 was determined to be 34.4%. In contrast, transfection efficiency in endothelial cells transfected with DMRIE-C or lipofectin was much lower, i.e. approximately 9.5 or 4.7%, respectively. In control cultures and in cultures exposed to the naked plasmid DNA, positive staining for hpAP was negligible. Figure 5B depicts HUVEC positively stained for the presence of alkaline phosphatase (arrows) as observed under the fluorescent microscope.



A



B

Fig. 5. A Efficiency of liposome-mediated transfection in HUVEC as measured by flow cytometry. Cells were transfected for 1.5 h with the VR-3301 vector (5 μg/ml) complexed with 40 μg/ml of DMRIE-C or lipofectin or with 36 μg/ml of pFx-7. *Values marked with an asterisk are significantly higher as compared to the values for control cultures or cultures transfected with naked plasmid DNA. †Values in cultures transfected in the presence of pFx-7 are significantly higher than values in other experimental groups. **B** An example of HUVEC positively stained for hpAP as observed under a fluorescent microscope (rhodamine filter). Transfection was mediated by pFx-7 under conditions as described in the legend to **A**.

Effectiveness of the Optimized Transfection Technique to Study Activation of Transcription Factors in HUVEC and for Transient Transfection of Different Endothelial Cell Types

One of the major applications of transient transfections is to study activation of transcription factors and mechanisms of gene regulation. Therefore, our optimized transfection technique (i.e., pFx-7, 36 μg/ml; plasmid DNA concentration, 5 μg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) was employed in such an experimental setting. HUVEC were transfected with NF-κB- or AP-1-responsive plasmids containing the firefly luciferase reporter gene, and luciferase activity was determined in cells stimulated with TNF (10 ng/ml) or LPS (1 μg/ml). The results of these experiments are shown in figure 6. Both TNF and LPS significantly increased luciferase activity in HUVEC transfected with NF-κB or AP-1-responsive plasmids. These data are consistent with TNF or LPS-induced activation of NF-κB or AP-1 in HUVEC, as determined by electrophoretic mobility shift assay (data not shown).

Structure and functions of endothelial cells originated from different tissues differ markedly [25]. Therefore, experiments were performed in which the optimized transfection technique was employed to compare transfection rates in different types of endothelial cells, namely in HUVEC, HAEC, HBMEC, and HMEC-1. The optimized transfection conditions (i.e., pFx-7, 36 μg/ml; pGL3,

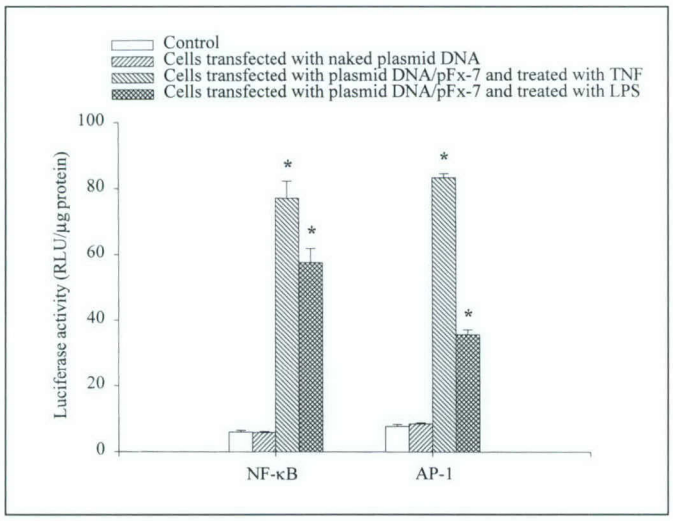


Fig. 6. The effectiveness of the optimized transfection conditions to study activation of transcription factors in HUVEC. Cells were transfected for 1.5 h with 5 µg/ml of the NF-κB or AP-1-responsive constructs complexed with 36 µg/ml of pFx-7. Transfections were followed by a 24-hour recovery period in normal growth medium, after which cells were treated either with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 24. Values are mean ± SEM. *Values marked with an asterisk are significantly higher as compared to those of control cultures or cultures transfected with naked plasmid DNA.

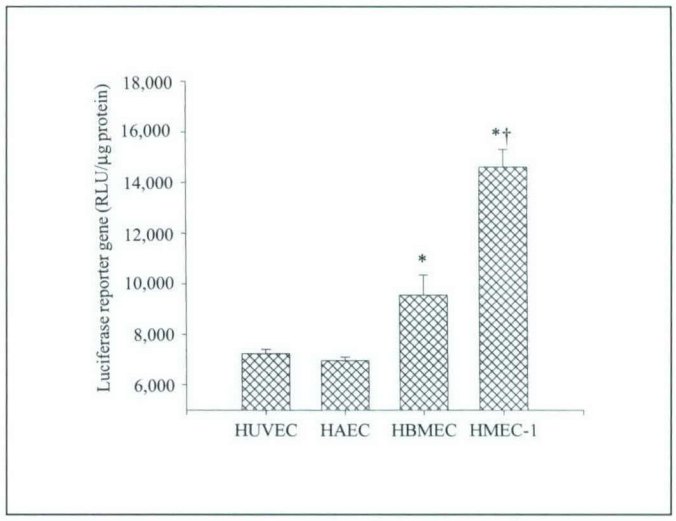


Fig. 7. A comparison of transfection rates in different types of human endothelial cells. HUVEC, HAEC, HBMEC, and HMEC-1 were transfected for 1.5 h with 5 µg/ml of the pGL3 vector complexed with 36 µg/ml of pFx-7. Luciferase activities in control (non-transfected) cells and in cells transfected with naked pGL3 were negligible and were not plotted. Values are mean ± SEM. *Transfection rates in HBMEC and HMEC-1 are significantly higher than those in HUVEC. †Transfection rates in HMEC-1 are significantly higher than those in other experimental groups.

5 µg/ml; transfection time, 1.5 h followed by a 48-hour recovery period) were employed in these experiments. As depicted in figure 7, among studied endothelial cell types, pFx-7 mediated the highest transfection rates in the immortalized endothelial cell lines, in particular in HMEC-1. There were no differences in transfection rates between HUVEC and HAEC.

Discussion

Cationic liposomes are positively charged lipids which can be mixed with negatively charged DNA to form lipid-DNA complexes. The most important advantages of mediating transfection with liposomes are that they are easy to prepare, they can transfer genes of various sizes and they are not infectious [6]. The most recognized disadvantage of liposome-mediated transfection is low efficiency of transfection. However, it is possible that the development of new generations of cationic lipids and transfection methods may overcome this limitation. In addition, better understanding of the mechanisms of liposome-mediated transfection may also contribute to the development of experimental methods which would allow for higher transfection efficiency [26].

Several factors can affect liposome-mediated transfection, including cell type, culture conditions, lipid composition of the liposomes, promoter type, reporter gene type, and amount of transfected plasmid DNA and DNA/lipid ratio. The dependency of transfection on the type of endothelial cells was observed in the present study. In the present study, we observed that transfection rates in HUVEC were approximately at the same rate as in HAEC but significantly lower as compared to immortalized endothelial cell lines (fig. 7). This is in agreement with a widely accepted phenomenon that cell lines are easier to transfect than primary cell cultures, such as HUVEC. However, it should be noted that endothelial cells, in general, are difficult to transfect. This may relate to the fact that endothelial cells represent a physiologic barrier against invasion of the vessels and underlying tissues by exogenous substances. During liposome-mediated transfection, lipids can fuse with cell membranes and thus deliver DNA into the cytoplasm. Liposome-mediated transfections are usually more efficient in dividing cells, because the nuclear membrane, which prevents DNA

from entering the nucleus, is not present during replication [6]. For this reason, transfections performed in the present study were initiated at approximately 55–65% confluency, i.e., in a state when cultured endothelial cells divide rapidly. However, it should be pointed out that liposomes can also transfect non-replicating cells [2].

Although liposome-delivered foreign DNA can enter the nucleus, it is not incorporated into the host genome. Therefore, liposome-mediated transfections are not mutagenic. The transfected plasmids remain as episomal nonreplicating minichromosomes and are gradually degraded [4]. In the present study, the reporter gene assays were performed 48 h following transfection, the standard interval for measuring reporter gene expression in cell cultures [4].

Because of the heterogeneity of cellular membranes, for optimal transfection, different types of cells require liposomes characterized by specific lipid profiles. In fact, lipid composition is the most critical factor determining the efficiency of liposome-mediated transfection. In the present study it was determined that among several commercially available liposomes pFx-7 is the most suitable lipid carrier for transfection of HUVEC. The optimal transfection rate was achieved when cells were incubated for 1.5 h with 36 μg pFx-7/ml complexed with 5 μg of plasmid DNA (fig. 2, 4). Although relatively high concentrations of pFx-7 induced cytotoxic effects in HUVEC, maintaining cells in normal growth medium for 48 h following transfection allowed for full recovery of morphological features (fig. 3). Among studied liposomes, incubation of endothelial cells with lipofectin resulted in most marked inhibition of endothelial cell proliferation (table 1). This is in agreement with an earlier report in which high toxicity of this liposome also was observed in cultured human endothelial cells [23].

In addition to comparing transfection efficiency in HUVEC mediated by different liposomes, transfections with the pGL3 vector alone were also included in the present study. It has been reported that injection with naked DNA plasmid encoding for VEGF into skeletal muscle was beneficial in patients with critical limb ischemia [14]. In addition, exposure of neurons to naked decoy κB DNA inhibited amyloid β-peptide-induced NF-κB activation [27]. However, in the present study transfection of HUVEC with naked DNA produced only a minimal effect. This is in agreement with the earlier report in which transfection efficiency with naked DNA plasmid was reported as low as approximately 0.08% [5].

Rates of liposome-mediated transfection are dependent on amounts of plasmid DNA and thus on the ratio of DNA/cationic lipids. Our studies revealed that the transfection rate of HUVEC can be enhanced with an increase in the amount of transfected DNA up to 5 μg DNA/ml (fig. 4). Further increases in the amount of plasmid DNA, and thus alteration of the DNA/liposome ratio, decreased efficiency of transfection. Similar results were obtained in the earlier studies [5]. Therefore, 5 μg DNA/ml was the standard amount of plasmid DNA used in the majority of the reported experiments.

The type of promoter which regulates the transgene expression can greatly influence transfection efficiency [28]. For example, using a plasmid regulated by the human β-actin promoter, it was reported that efficiency of transfection of HUVEC by electroporation was approximately 0.68%, by lipofectin approximately 0.45%, and by other transfection methods, including calcium phosphate and DEAE-dextran-mediated transfection, also below 1% [29]. In contrast, lipofectin-mediated transfection of HUVEC with a plasmid regulated by a strong respiratory syncytial virus (RSV) viral promoter resulted in transfection efficiency as high as 10–20% [30]. Highly efficient transfection of approximately 20% was also achieved in HUVEC transfected with a plasmid regulated by the CMV promoter, using γAP-DLRIE/DOPE liposomes [5]. Constructs employed in the present study also contained strong promoters. The pGL3 vector is regulated by the SV40 promoter and the VR-3301 vector contains the CMV promoter. Because these strong promoters use transcription factors which are present in host cells, they can be constitutively active in transfected cells. For example, the CMV promoter contains binding elements for common transcription factors, such as cyclic adenosine monophosphate and NF-κB [2]. These transcription factors remain active at the baseline level even in non-stimulated cells. In addition, one may suggest that cellular stress connected with transfection may further stimulate activation of these transcription factors. It should be noted that the CMV promoter can provide better transfection rates in HUVEC compared to the RSV promoter. This was demonstrated in experiments in which HUVEC were transfected with plasmids encoding for the same reporter gene (hpAP) but driven either by the CMV or the RSV promoter [5].

In the present study, transfection conditions were optimized using the pGL3 vector regulated by the SV40 promoter and encoding for firefly luciferase. Firefly luciferase has been recognized to be the reporter gene of choice for transfection studies in cells resistant to uptake of foreign DNA [31]. The transgene is simple to measure and has no background levels in animal tissues. In contrast,

our preliminary experiments with β-galactosidase revealed background activity of this enzyme in cultured HUVEC (data not shown). Determination of luciferase activity also has the advantage of being several orders of magnitude more sensitive than other common reporter gene assays, such as activities of chloramphenicol acetyltransferase, β-galactosidase or alkaline phosphatase [4, 31]. However, to determine the efficiency of transfection, the vector encoding for hpAP was used. This experimental approach allowed us to stain and count the transfected cells. Transfection efficiency of 32% achieved in HUVEC in the present study is higher than in earlier studies which reported efficiencies of approximately 20% [5, 30]. However, it should be noted that much higher transfection efficiency can be achieved for liposome-mediated transfection of endothelial cells with antisense oligonucleotides. For example, it was reported that cytofectin GCV or SuperFect can mediate the uptake of antisense oligonucleotides to more than 95% of cultured human iliac artery endothelial cells [23]. In contrast, these liposomes appeared to be less effective in facilitation of transfection of plasmids, such as the pGL3 vector, into HUVEC (data not shown).

In the present study, a strong correlation between transfection rates and transfection efficiency was observed. The high transfection rates mediated by pFx-7 were associated with high transfection efficiency in endothelial cells transfected in the presence of this liposome. In contrast, transfections mediated by either DMRIE-C or lipofectin resulted in moderate transfection rates and efficiency.

In summary, efficient transfection conditions have been established for a transient transfection of human endothelial cells. The optimal transfection conditions, resulting in the transfection efficiency of approximately 32%, were achieved with cationic liposome pFx-7 used at the concentration of 36 μg/ml for 1.5 h. Although these transfection conditions were connected with some cytotoxicity, a 48-hour period of maintaining endothelial cells in normal growth medium allowed the cells to recover fully. We conclude that pFx-7 can be used as an efficient transfection agent to deliver foreign DNA into human endothelial cells.

Acknowledgements

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Fatty Acid–Mediated Activation of Vascular Endothelial Cells

Bernhard Hennig, Purushothaman Meerarani, Pachaikani Ramadass, Bruce A. Watkins, and Michal Toborek

Vascular endothelial cell activation and dysfunction are critical early events in atherosclerosis. Selected dietary lipids (eg, fatty acids) may be atherogenic by activating endothelial cells and by potentiating an inflammatory response. Due to their prooxidant property, unsaturated fatty acids may play a critical role in endothelial cell activation and injury. To test this hypothesis, porcine endothelial cells were exposed to 18-carbon fatty acids differing in the degree of unsaturation, ie, 90 μmol/L stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), or linolenic acid (18:3n-3) for 6 to 24 hours and/or tumor necrosis factor alpha ([TNF-α] 500 U/L) for up to 3 hours. Compared with control cultures, treatment with 18:0 and 18:2 decreased glutathione levels, suggesting an increase in cellular oxidative stress. Both 18:2 and 18:0 activated the transcription factor nuclear factor κB (NF-κB) the most and 18:1 the least. This NF-κB–dependent transcription was confirmed in endothelial cells by luciferase reporter gene assay. The fatty acid–mediated activation of NF-κB was blocked by preenrichment of the cultures with 25 μmol/L vitamin E. All fatty acids except 18:1 and 18:3 increased transendothelial albumin transfer, and 18:2 caused the most marked disruption of endothelial integrity. Preenrichment of endothelial cells with 18:2 followed by exposure to TNF-α resulted in a 100% increase in interleukin-6 (IL-6) production compared with TNF-α exposure alone. In contrast, cellular preenrichment with 18:0, 18:1, or 18:3 had no effect on TNF-α–mediated production of IL-6. Cellular release of radiolabeled arachidonic acid (20:4) was markedly increased only by cell exposure to 18:2 and 18:3, and the release of 20:4 appeared to be mainly from the phosphatidylethanolamine fraction. These data suggest that oleic acid does not activate endothelial cells. Furthermore, linoleic acid and other omega-6 fatty acids appear to be the most proinflammatory and possibly atherogenic fatty acids.

EVIDENCE SUGGESTS that the mechanisms of vascular disease such as atherosclerosis involve damage to the endothelium, which then reduces its effectiveness as a selectively permeable barrier to plasma components.[1,2] The endothelium interacts with the blood and underlying tissues, serves as both a prothrombotic and antithrombotic surface, and releases regulatory factors important in modulating vascular tone. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium induced by numerous activating molecules, such as certain lipids, prooxidants, and inflammatory cytokines. These risk factors may contribute to an overall cellular imbalance of the oxidative stress/antioxidant balance, thus leading to chronic activation or stimulation of the endothelium, as well as endothelial barrier dysfunction, which can result in accelerated uptake of cholesterol-rich lipoproteins into the vessel wall.

There is ample evidence suggesting that serum cholesterol is a predictor of atherosclerosis and that serum cholesterol concentrations can be modified by varying the composition of dietary fat. However, less is known about the role of specific fatty acids in atherosclerosis. The role of saturated fatty acids in atherosclerosis has been questioned recently.[3-5] In fact, data obtained in subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels (eg, linoleic acid), when insufficiently protected by antioxidants (eg, vitamin E), may indicate a higher risk of atherosclerosis.[6] Recent research with a population from a country with one of the highest dietary polyunsaturated to saturated fat ratios in the world has concluded that diets rich in n-6 (or omega-6) fatty acids may contribute to an increased incidence of atherosclerosis, hyperinsulinemia, and tumorigenesis.[7]

A transcription factor implicated in many endothelial cell activation responses to injury and stress is nuclear factor κB (NF-κB).[8,9] NF-κB plays a central role in regulating the cytokine network, and hence its activation may be a major factor in the pathogenesis of atherosclerosis. NF-κB can be activated by a variety of pathogenic or pathogen-elicited stimuli including cytokines, lipids, mitogens, bacteria, and related products, with the common denominator apparently being reactive oxygen species. Many target genes in endothelial cells contain NF-κB or NF-κB–like binding sites in the promoter genes coding for inflammatory cytokines (eg, tumor necrosis factor [TNF] and interleukin-6 [IL-6] and adhesion molecules).[10]

In light of the evidence that oxidative stress plays a critical role in atherosclerosis[11,12] and that antioxidant nutrients such as vitamin E may provide protection against this disease,[13,14] one may speculate that the atherosclerotic risk of dietary lipids may be directly related to their degree of unsaturation. Thus, a focus of the present study was to examine the mechanisms of the effects of 18-carbon fatty acids, differing in degree of unsaturation, on endothelial cell activation.

MATERIALS AND METHODS

Cell Culture and Experimental Media

Porcine pulmonary artery–derived endothelial cells were isolated from porcine pulmonary arteries and cultured as described previously.[15]

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Cells were subcultured in medium 199 (M-199) containing 10% bovine calf serum (HyClone Laboratories, Logan, UT) using standard techniques. The purity of the cultures was determined by morphological criteria and by quantitatively measuring angiotensin-converting enzyme activity, or by the uptake of fluorescent-labeled acetylated low-density lipoprotein (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes, Eugene, OR).

The experimental media were composed of M-199 enriched with 5% FBS and either fatty acids (90 μmol/L) or TNF-α (500 U/mL or 100 ng/mL; Knoll Laboratories, Whippany, NJ). Fatty acids (>99% pure) were obtained from Nu-Chek Prep (Elysian, MN). Preparations of experimental media with fatty acids and/or TNF were made as described previously.[15,16] Thus, fatty acids were introduced into the media bound to serum albumin. Assuming albumin concentrations of 30 μmol/L (in 5% serum) to 60 μmol/L (in 10% serum) in our culture media, the fatty acid concentrations are within physiological and metabolic relevance. Even though only about 5% of total free fatty acids in the experimental media are derived from the serum, fatty acid–mediated activation of endothelial cells may vary depending on the type of serum in which cells are cultured.[17] For most experimental settings, cells were treated with fatty acids for 6 to 24 hours and/or TNF for 3 hours before termination. Some cultures were preenriched with 25 μmol/L vitamin E (α-tocopherol). All experimental outcomes were confirmed more than twice.

Glutathione Assay

Glutathione assays were performed according to a modified method of Baker et al.[18] To determine total glutathione, cellular protein was precipitated by adding 100 μL ice-cold 0.09% sulfosalicylic acid (SSA) to cells collected from P-100 tissue culture plates. The culture plates were then incubated at 40°C for 15 minutes, after which the cell lysates were collected and centrifuged at 9,000× *g* for 5 minutes. Glutathione levels were determined spectrophotometrically using the glutathione-linked 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) recycling assay. The mixture for the assay contained 50 μL supernatant and 100 μL 125-mmol/L phosphate buffer containing 0.225 mmol/L DTNB, 0.302 mmol/L NADPH, and glutathione reductase at a concentration of 1.25 U/μL. The blank contained 50 μL 0.09% 5-SSA instead of the supernatant, and the control reaction contained the glutathione standard in place of the supernatant. The mixtures were equilibrated at room temperature for 3 minutes, and the reaction was started by the addition of 100 μL reaction buffer. Absorbance was measured at 405 nm in a 96-well plate reader.

Transcription Factor (NF-κB) Activation Studies: Electrophoretic Mobility Shift Assay

These transcription factors, which bind to enhancer elements on DNA, were determined in endothelial cells by an electrophoretic mobility shift assay as described by Sen and Baltimore.[19] Nuclear extracts containing the NF-κB active protein were prepared from cells according to the method of Dignam et al.[20] Nuclear extracts were incubated for 20 to 30 minutes with ^{32}P-end-labeled oligonucleotide probe (GIBCO/BRL, Gaithersburg, MD) containing the κB enhancer DNA element with a tandem duplicate of a NF-κB binding site (-GGGGACTTTCC-). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 5% low–ionic-strength nondenaturing polyacrylamide gel and visualized by autoradiography. Control reactions using a 200-fold molar excess of unlabeled oligonucleotide probes or a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF-κB.

Transfection and Luciferase Assay

The luciferase reporter gene assay reflects NF-κB–dependent transcription. Briefly, endothelial cells were transfected with 2 μg pNF-κB-Luc plasmid (Stratagene, La Jolla, CA) by the lipofection method (Invitrogen, Carlsbad, CA). Four hours after transfection, cells were washed with phosphate-buffered saline (PBS) and incubated with M-199 (with 10% serum) for 24 hours. Then, the endothelial cells were stimulated with 90 μmol/L fatty acid (18:0 or 18:2) for 24 hours. Luciferase activity was determined following the instructions described in the luciferase assay kit (Promega, Madison, WI) using a luminometer.

IL-6 Production

After exposure to fatty acids and TNF, the media were removed from the wells and frozen immediately at −80°C until IL-6 analysis. The remaining cells were trypsinized and washed with PBS twice and resuspended in 0.2% sodium dodecyl sulfate with 0.2 mol/L NaOH for protein analysis.[21] IL-6 production and release into the medium was determined using the murine hybridoma cell line B9 (kindly supplied by Dr L.A. Aarden, Emeryville, CA) as described by Helle et al.[22] The B9 cell line viability is IL-6–dependent, and thus, the incorporation of ^{3}H-thymidine by viable cells is a reflection of the quantity of IL-6 produced by endothelial cells.

Endothelial Barrier Function (albumin transfer studies)

Endothelial barrier function was measured as transendothelial albumin transfer using polystyrene chambers with a 0.8-μm pore size polycarbonate membrane (Millipore, Bedford, MA) as described previously.[15] After achieving approximate confluence, endothelial monolayers were exposed to control or experimental media for 24 hours. Following treatments, chambers with endothelial cells attached to the membranes were washed with M-199 and exposed to 200 μmol/L bovine serum albumin (fatty acid–free; Sigma Chemical, St Louis, MO) in M-199 for 1 hour. After incubation, the albumin transferred across endothelial monolayers was determined using bromcresol green (Sigma) and recorded spectrophotometrically at 630 nm.

Lipid Analysis

Measurement of arachidonic acid release. Endothelial cells were cultured in M-199 enriched with 10% FBS and incubated with ^{3}H-arachidonic acid (0.2 mCi/mL medium) for 24 hours. Following incubation with radiolabeled 20:4, the cells were washed with serum-free M-199 medium and medium supplemented with 0.2% fatty acid–free BSA and then exposed to different 18-carbon fatty acids (90 μmol/L) for 6 hours. Subsequently, the media were collected and centrifuged at 3,000 rpm for 10 minutes to remove floating cells, and radioactivity was measured in the supernatant. The cells were immediately scraped in PBS, and lipids were extracted with chloroform: methanol (2:1) using a modified method of Takenaka et al.[23]

Separation of arachidonic acid and phospholipid fractions. Lipid extracts from each treatment were applied to a silica gel thin-layer chromatography (TLC) plate, and the separation of arachidonic acid and different phospholipids was performed using chloroform:methanol: ammonia (65:25:4) as a mobile phase. After identification of lipids in iodine vapor, arachidonic acid and phospholipid spots were scraped from the plate into scintillation vials with 10 mL scintillation cocktail (3a70B). The radioactivity of the samples was measured in a Tri-Carb2100TR liquid scintillation analyzer (Packard Instrument, Meriden, CT).

Statistical Analysis

Data were analyzed statistically using a 1-way ANOVA. For each endpoint, the treatment means were compared in pairs using the

Bonferroni procedure.[24] A P value of .05 or less was considered significant.

RESULTS

The effects of the 18-carbon fatty acids on cellular redox status were determined by measurement of cellular glutathione levels. Figure 1 demonstrates that both 18:0 and 18:2 significantly decreased glutathione levels. Compared with control cultures, treatment with 18:1 increased total glutathione, whereas 18:3 had no effect on intracellular glutathione levels.

The evidence suggests that oxidative stress can affect cellular metabolism by an increased expression of genes regulated by NF-κB. Interestingly, 18:0, the only saturated fatty acid, and 18:2 activated the transcription factor NF-κB most markedly (Fig 2), whereas 18:1 exposure to endothelial cells had little effect on the activation of this transcription factor. To test whether vitamin E can protect against fatty acid–induced activation of NF-κB, endothelial cells were pretreated with vitamin E for 24 hours before coexposure to fatty acids for an additional 6 hours (Fig 3). Vitamin E markedly decreased NF-κB binding induced by 18:0 or 18:2.

To determine whether 18:0- or 18:2-activated NF-κB can induce gene expression, endothelial cells were transfected with a plasmid (pNF-κB-Luc) encoding the bacterial protein luciferase. The expression of this construct is controlled by a promoter responsive to NF-κB. Results of the luciferase reporter gene assay are shown in Fig 4. Both 18:0- and 18:2-mediated activation of NF-κB were sufficient to induce NF-κB–dependent transcription in cultured endothelial cells. Compared with control cultures, luciferase activity was significantly higher in both 18:0- and 18:2-treated cells.

Figure 5 shows the effect of cellular incubation with control medium and media enriched with 18-carbon fatty acids on endothelial barrier function. Compared with control cultures, all fatty acids except 18:1 and 18:3 increased albumin transfer across endothelial monolayers. However, treatment with 18:2 disrupted endothelial barrier function most markedly.

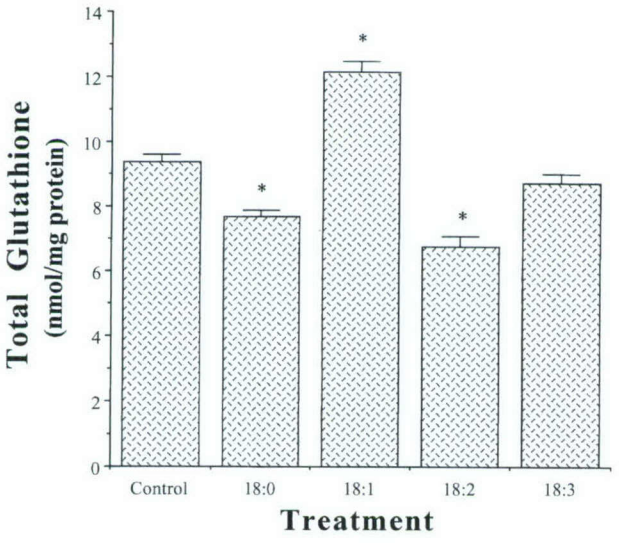

Fig 1. Effect of treatment with different 18-carbon fatty acids (90 μmol/L) on total glutathione levels in cultured endothelial cells. Cells were exposed to experimental media for 6 hours. Values are the mean ± SEM (n = 3). *Significantly different *v* control cultures.

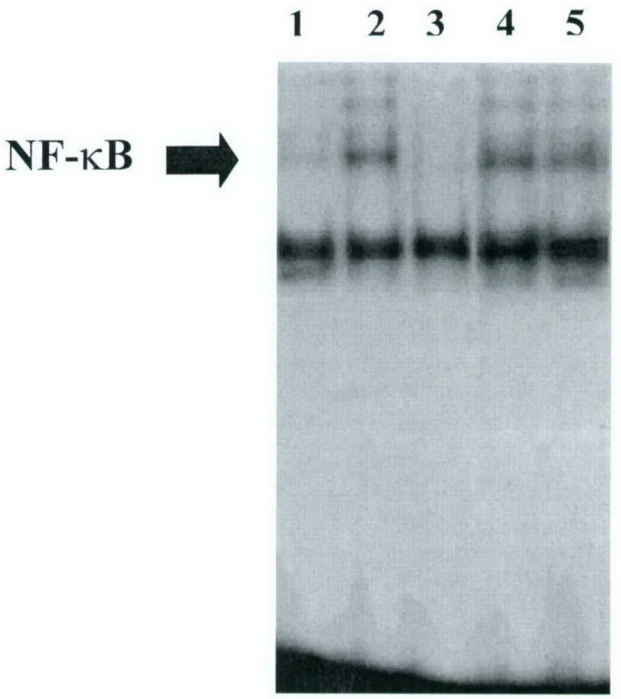

Fig 2. Effect of different 18-carbon fatty acids on activation of NF-κB. Cells were treated with the different fatty acids (90 μmol/L) for 6 hours. Lane 1, control; lane 2, stearic acid (18:0); lane 3, oleic acid (18:1); lane 4, linoleic acid (18:2); lane 5, linolenic acid (18:3). The specific binding of NF-κB was confirmed by both competitive (excess unlabeled oligonucleotide) and supershift assays.

Figure 6 shows IL-6 production in endothelial cells during fatty acid treatment for 9 hours followed by TNF exposure for an additional 3 hours. These data show that the cellular lipid environment can modify TNF-mediated inflammatory properties by selectively promoting endothelial cell–mediated production of IL-6. Compared with TNF treatment alone, preenrichment of endothelial cells with 18:2 followed by exposure to TNF resulted in a 100% increase in IL-6 production. In contrast, cellular preenrichment with 18:0, 18:1, and 18:3 had no further effect on the TNF-α–mediated production of IL-6.

The fatty acid–mediated changes in oxidative stress and other observed mediators of endothelial cell activation may be due to an increase in phospholipase A_2 activity and thus an increase in available arachidonic acid (20:4n-6) for metabolic activity. To test this hypothesis, cells were preenriched with radiolabeled 20:4 for 24 hours, carefully washed, and then treated with the 18-carbon fatty acids for an additional 6 hours. The surrounding media then were tested for cellular release of radiolabeled 20:4 (Fig 7). Cells were also harvested and analyzed for radioactivity in various lipid fractions, including phospholipids (Fig 8). Compared with control cultures (cells not enriched with 18-carbon fatty acids), cellular release of radiolabeled 20:4 was markedly increased only by cell exposure to 18:2 or 18:3 (Fig 7). Neither 18:0 nor 18:1 affected 20:4 release. Preenriching cultures with vitamin E decreased the fatty acid–mediated release of 20:4 into the media in all cultures independently of

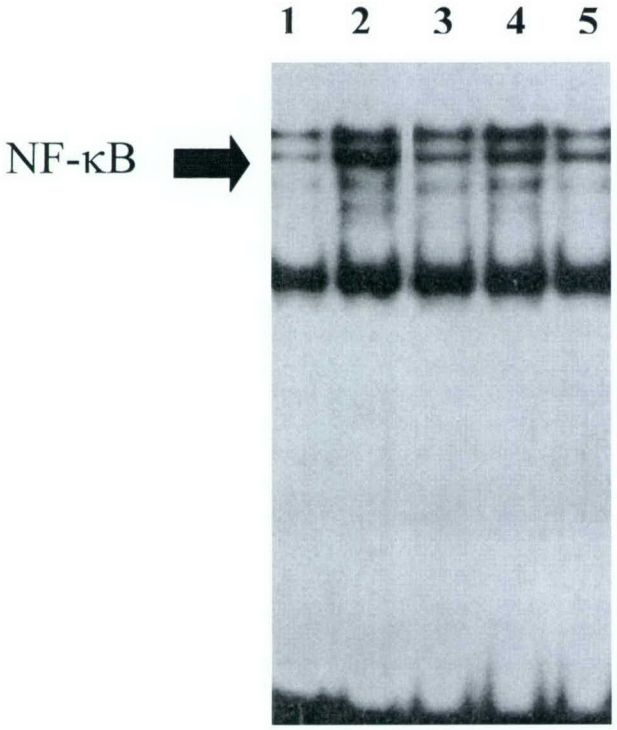

Fig 3. Effect of preenrichment with vitamin E on fatty acid–mediated activation of NF-κB. All cells were exposed to the different fatty acids (90 μmol/L) for 6 hours, and some cultures were first preenriched with vitamin E for 24 hours. Lane 1, control + vitamin E; lane 2, stearic acid (18:0); lane 3, 18:0 + vitamin E; lane 4, linoleic acid (18:2); lane 5, 18:2 + vitamin E.

the type of 18-carbon fatty acid to which the endothelial cells were exposed (data not shown). When analyzing for radiolabeled 20:4 in several types of cellular phospholipids, only its level in the phosphatidylethanolamine fraction was affected to a significant extent by 18-carbon fatty acid treatment. The most marked decrease in 20:4 incorporation into this phospholipid fraction was in cultures treated with 18:2, followed by cultures treated with 18:3. Neither 18:0 nor 18:1 treatment affected the 20:4 content in the phosphatidylethanolamine fraction. Thus, it appears that treatment with 18:2 or 18:3 can stimulate the release of 20:4 specifically from phosphatidylethanolamine.

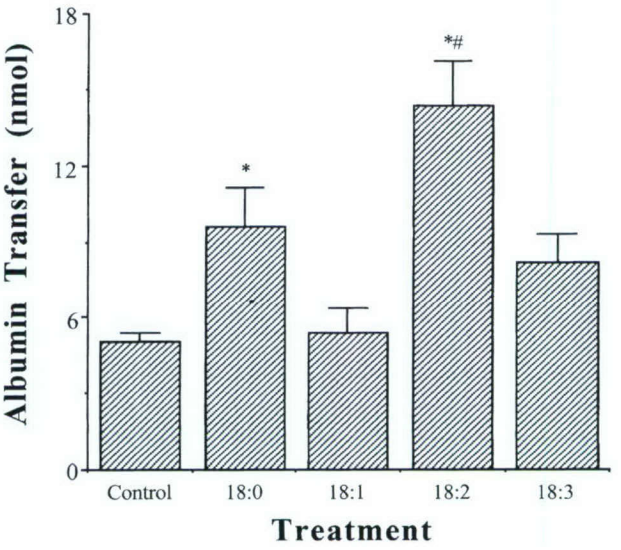

Fig 5. Effect of fatty acid exposure on albumin transfer across endothelial monolayers. Cultures were exposed to different 18-carbon fatty acids (90 μmol/L) for 24 hours. Subsequently, albumin transfer was measured over a 1-hour period. Values are the mean ± SEM (n = 6). *Significantly higher *v* control cultures. #Significantly higher *v* cultures treated with 18:0.

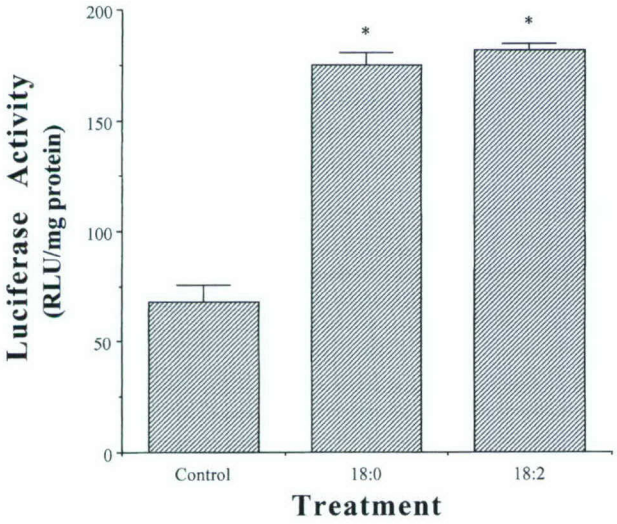

Fig 4. Effect of a 24-hour exposure to 18:0 or 18:2 on NF-κB–dependent transcription as measured by luciferase reporter gene assay. Data are expressed as relative light units (RLU) per mg protein. Values are the mean ± SEM (n = 3). *Significantly different *v* control cultures.

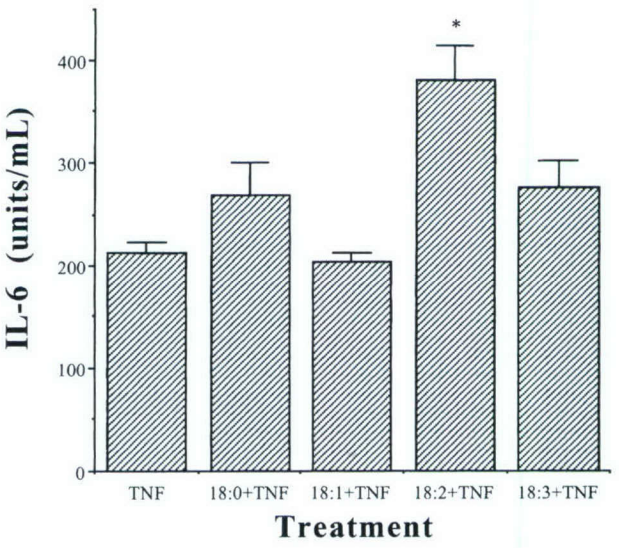

Fig 6. IL-6 production in endothelial cells after exposure to different 18-carbon fatty acids. Endothelial cells were treated with the different fatty acids (90 μmol/L) for 9 hours and with added TNF-α (500 U/mL) for an additional 3 hours. Values are the mean ± SEM (n = 3). *Significantly higher *v* control cultures.

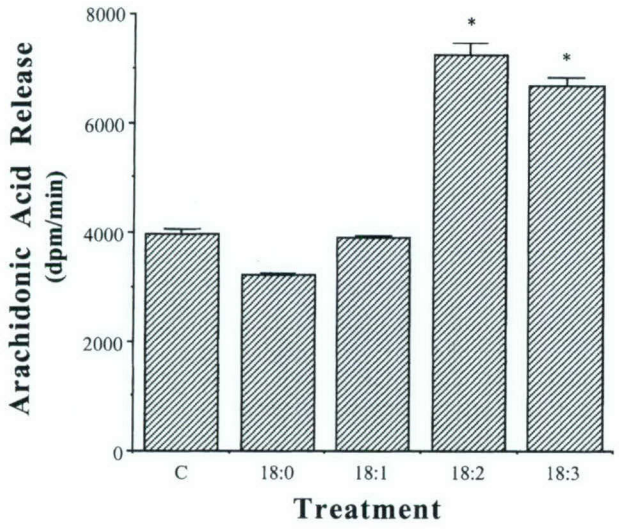

Fig 7. Release of radiolabeled 20:4 from endothelial cells following exposure to 18-carbon fatty acids (90 μmol/L) for 6 hours (cells were labeled with ^{3}H-arachidonic acid for 24 hours prior to fatty acid exposure). The media were collected and radioactivity was counted and expressed as dpm/min. Values are the mean ± SEM (n = 3). *Significantly higher *v* control cultures.

DISCUSSION

Although the mortality from coronary heart disease has declined recently, atherosclerosis and related vascular disorders still are the leading cause of death in the United States and other Western countries. Injury to or abnormal mechanisms of the vascular endothelium may be initiating events in the etiology of atherosclerosis. Dietary fat affects plasma lipids and lipoproteins and thus is linked to atherosclerosis.[25] The question then arises as to whether dietary saturated fats should be replaced by unsaturated fats. Unsaturated fats, especially monounsaturated[26,27] and n-3 or omega-3[28,29] fatty acids, may be beneficial to human health. However, replacing saturated lipids with unsaturated and especially polyunsaturated lipids may not be desirable because of their ability to oxidize easily. The evidence supports the hypothesis that low-density lipoprotein undergoes oxidative modifications that increase its uptake by macrophages.[11] In fact, data from subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels, when insufficiently protected by antioxidants (eg, vitamin E), may indicate a higher risk of atherosclerosis.[30]

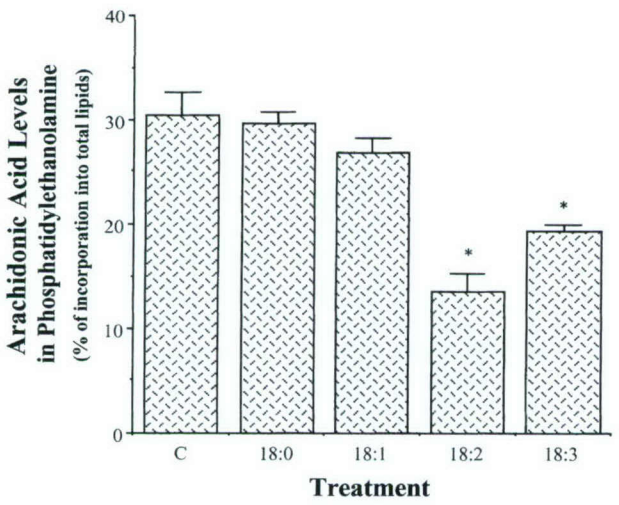

Fig 8. Incorporation of radiolabeled 20:4 into phosphatidylethanolamine. The experimental setup was the same as described in Fig 7. Lipids were obtained from total cell extracts and separated by TLC. Radioactivity was counted and expressed as dpm/min. Values are the mean ± SEM (n = 3). *Significantly lower *v* control cultures.

High levels of circulating triglyceride-rich lipoproteins (chylomicrons and very–low-density lipoprotein [VLDL]) have been implicated in the injury process of the endothelium.[31,32] Plasma chylomicron levels are elevated in humans after consuming a high-fat meal, and hepatic synthesis of VLDL is increased when the caloric intake is in excess of body needs. The hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism that is associated with the luminal site of endothelial cells, may be an important source of high concentrations of fatty acid anions in the proximity to the endothelium.[33] It has been hypothesized that high levels of diet-derived fatty acids can cause endothelial injury or dysfunction and thus disrupt the ability of the endothelium to function as a selective barrier.[33,34] This would result in lipid deposition by allowing increased penetration of cholesterol-rich remnant lipoproteins into the arterial wall. In fact, the activity of lipoprotein lipase is increased in atherosclerotic lesions.[35,36] A recent report also provides evidence that lipoprotein lipase may be a chemoattractant for activated macrophages.[37] Lipoprotein lipase–derived remnants of lipoproteins isolated from hypertriglyceridemic subjects, as well as selective unsaturated fatty acids such as linoleic acid, were demonstrated to disrupt endothelial integrity.[38,39] In fact, a recent study has provided the first evidence that the lipolytic remnant products of triglyceride-rich lipoproteins produced after a meal rich in polyunsaturated fat are more injurious to arterial wall cells than those produced after a meal rich in saturated fat.[40] Furthermore, activated lipoprotein lipase induces TNF gene expression in macrophages and TNF production by this type of cell.[41] Thus, endothelial cells may be simultaneously exposed to free fatty acids and TNF.

As mentioned before, there is evidence that selected fatty acids, derived from the hydrolysis of triglyceride-rich lipoproteins, may be atherogenic by causing endothelial injury or dysfunction and subsequent endothelial barrier dysfunction.[42] In support of this hypothesis, we again confirm in the present study that, compared with all 18-carbon fatty acids, 18:2 disrupted endothelial barrier function most markedly. These findings agree with our earlier findings that when comparing fatty acid extracts derived from different animal fats and plant oils, the fat-induced disruption of endothelial barrier function was related to the amount of 18:2 present in the fat source.[43] These data suggest that among different fatty acids, linoleic acid may play a critical role in the pathogenesis of atherosclerosis.[44] This hypothesis is supported by the fact that adipose tissue levels of 18:2, which reflect the intake of this fatty acid over time, were positively associated with the degree of coronary artery disease.[45] In addition, concentrations of 18:2 were increased in the phospholipid fractions of human coronary

arteries in cases of sudden cardiac death due to ischemic heart disease.[46]

Several mechanisms were proposed to explain the injurious effects of 18:2 to endothelial cells. Due to the very low basal activity of endothelial cell elongases and delta 5 and delta 9 desaturases, arachidonic acid is not produced from 18:2 significantly in this type of cell.[47,48] Consequently, 18:2 accumulates within endothelial cells.[47,49] Moreover, 18:2 decreases the level of intracellular ATP[50] and proteoglycans,[51] enhances elastase-like activity,[52] and can yield nitrated oxidation species by reacting with nitric oxide–derived products.[53] The 18:2-mediated disruption of endothelial barrier function also may be caused by its ability to inhibit gap-junctional intracellular communication[54,55] and to induce intracellular oxidative stress.[40] Furthermore, 18:2, but not 18:0, can activate phospholipase A_2, as measured by the cellular release of 20:4 in neutrophils.[56] In fact, polyunsaturated free fatty acids that are liberated by phospholipase A_2 increased the formation of bioactive phospholipids in LDL, which stimulated endothelial cell activation and monocyte–endothelial cell interactions.[57]

In recent years, the role of oxidative stress has gained much attention in studies of lipid- and/or inflammatory cytokine–mediated endothelial cell dysfunction or injury. It is now generally accepted that LDL oxidation plays one of the most critical roles in atherogenesis. LDL can be oxidized in the subendothelial space, which lacks many of the antioxidants present in the whole blood. Furthermore, dietary oxidized lipids can be absorbed by the small intestine, be incorporated into chylomicrons, appear in the bloodstream, and thus contribute to the total body pool of oxidized lipids.[58] Including oxidized corn oil (a rich source of 18:2) in a diet accelerated the development of fatty streaks in cholesterol-fed rabbits,[59] suggesting that the consumption of oxidized lipids (eg, high–corn oil diets) may be an important risk factor for atherosclerosis. Our data support the notion that omega-6 fatty acids, and especially fats rich in 18:2, are atherogenic by activating vascular endothelial cells and by promoting an inflammatory response. We clearly show that 18:2 most markedly amplifies TNF-mediated IL-6 production by endothelial cells. An increase in oxidative stress and subsequent activation of NF-κB may be one of the main mechanisms of the inflammatory properties of 18:2. However, there appears to be no relationship between the degree of unsaturation of fatty acids and endothelial cell activation. In fact, stearic acid (18:0) appears to activate endothelial cells more markedly than either 18:1 or 18:3. Furthermore, 18:1 had little or no effect on endothelial cell activation. Interestingly, when studying lipoproteins from subjects consuming different types of dietary fat, eg, oleic acid or linoleic acid, only the percentage of 18:2 in LDL correlated strongly with the extent of oxidizability and macrophage degradation of these lipoproteins.[60]

It is not clear why 18:0 decreased cellular glutathione and increased NF-κB activation so markedly. Although 18:0, as a saturated fatty acid, does not undergo peroxidative modifications, it may induce perturbations in cellular metabolism, which secondarily can result in oxidative stress and be responsible for the observed decreases in glutathione concentrations. On the other hand, 18:0 may influence gene expression or signal transduction pathways that are more substantial than its unknown or secondary effects on oxidative stress. The fact that preenrichment of cultures with vitamin E can block the activation of NF-κB suggests that this fatty acid can modify the cellular lipid milieu, leading to an imbalance in oxidative stress/antioxidant status and to endothelial cell activation. Because of its lack of double bonds, 18:0 may affect the membrane properties of endothelial cells differently compared with fatty acids with *cis* double bonds. 18:0 also may be taken up and metabolized differently than fatty acids that contain double bonds. In fact, once taken up by endothelial cells, 18:0 is randomly distributed among membrane phospholipids,[61] whereas unsaturated fatty acids are initially preferentially incorporated into phosphatidylcholine and then can undergo a time-dependent transfer to phosphatidylethanolamine.[61] Furthermore, using electron-spin resonance studies, we found that of all 18-carbon fatty acids, only 18:0 increased membrane fluidity.[62] In that same study, a relationship between membrane fluidity and fatty acid compositional alterations in cellular phospholipids was observed, ie, only the unsaturated fatty acids, not 18:0, decreased the cellular arachidonic acid content. These and our present data suggest that 18:0 may have unique membrane-modifying effects.

In summary, our data suggest that omega-6 fatty acids appear to be most effective in activating endothelial cells and in contributing to an inflammatory response. In contrast, 18:1 does not appear to activate endothelial cells, and in fact may protect endothelial cells against oxidative insult.[63] These data support the concept that the substitution of dietary monounsaturated fatty acids and not polyunsaturated fatty acids for saturated fatty acids might be preferable for the prevention of cardiovascular disease.

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